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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2004904834 for a patent by MONASH UNIVERSITY as filed on 24 August 2004.

WITNESS my hand this
Fourteenth day of October 2004

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Regulation 3.2

Monash University

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Therapeutic Method - II"

The invention is described in the following statement:

THERAPEUTIC METHOD - II

FIELD OF THE INVENTION

The present invention relates generally to a method of modulating an inflammatory response in a mammal and to agents useful for same. More particularly, the present invention relates to a method of modulating an inflammatory response in a mammal by modulating the functional activity of activin and thereby modulating the pro-inflammatory mediator cascade. The method of the present invention is useful, *inter alia*, in the treatment and/or prophylaxis of conditions characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response including, *inter alia*, sepsis and inflammation of the airway. The present invention is further directed to methods for identifying and/or designing agents capable of modulating activin mediated regulation of the inflammatory response.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Mammals are required to defend themselves against a multitude of pathogens including viruses, bacteria, fungi and parasites, as well as non-pathogenic insults such as tumours and toxic, or otherwise harmful, agents. In response, effector mechanisms have evolved which are capable of mounting a defence against such antigens. These mechanisms are mediated by soluble molecules and/or by cells.

In the context of these effector mechanisms, inflammation is a complex multifaceted process in response to disease or injury which is regulated by the release of a series of cytokines (Alexander et al, 2001, *J Endotoxin Res* 7:167-202). These cytokines are classified in general terms as pro- or anti-inflammatory cytokines and the critical balance between release and activity of cytokines with opposing actions regulates the inflammatory response to prevent it from becoming overt or understated.

If the inflammatory response continues unchecked and is overt then the host may suffer associated tissue damage and in severe cases this may present as septic shock and multi-organ failure can occur (Ulevitch *et al.*, 1999, *Curr Opin Immunol* 11:19-22). Conversely, a poor or understated inflammatory response may mean uncontrolled infection resulting in chronic illness and host damage. regulation of the inflammatory response is important at both the systemic level and the local level.

The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response. There are five basic indicators of inflammation, these being redness (rubor), swelling (tumour), heat (calor), pain (dolor) and deranged function (functio laesa). These indicators occur due to extravasation of plasma and infiltration of leukocytes into the site of inflammation. Consistent with these indicators, the main characteristics of the inflammatory response are therefore:

(i) vasodilation – widening of the blood vessels to increase the blood flow to the infected area;

(ii) increased vascular permeability – this allows diffusible components to enter the site;

(iii) cellular infiltration – this being the directed movement of inflammatory cells through the walls of blood vessels into the site of injury;

(iv) changes in biosynthetic, metabolic and catabolic profiles of many organs; and

(v) activation of cells of the immune system as well as of complex enzymatic systems of blood plasma.

The degree to which these characteristics occur is generally proportional to the severity of the injury and/or the extent of infection.

The inflammatory response can be broadly categorised into several phases. The earliest, gross event of an inflammatory response is temporary vasoconstriction, i.e. narrowing of blood vessels caused by contraction of smooth muscle in the vessel walls, which can be seen as blanching (whitening) of the skin. This is followed by several phases that occur over minutes, hours and days later, as follows:

(i) The acute vascular response follows within seconds of a tissue insult and lasts for some minutes. It is characterised by vasodilation and increased capillary permeability due to alterations in the vascular endothelium, leading to increased blood flow (hyperaemia) that causes redness (erythema) and the entry of fluid into the tissues (oedema).

(ii) If there has been sufficient damage to the tissues, or if infection has occurred, the acute cellular response takes place over the next few hours. The hallmark of this phase is the appearance of granulocytes, particularly neutrophils, in the tissue. These cells first attach themselves to the endothelial cells within the blood vessels (margination) and then cross into the surrounding tissue (diapedesis). If the vessel is damaged, fibrinogen and fibronectin are deposited at the site of injury, platelets aggregate and become activated and clot formation occurs.

(iii) If damage is sufficiently severe, a chronic cellular response may follow over the next few days. A characteristic of this phase of inflammation is the appearance of a mononuclear cell infiltrate composed of macrophages and lymphocytes. The macrophages are involved in microbial killing, in clearing up cellular and tissue

debris, and are also thought to play a significant role in remodelling tissue.

(iv) Over the next few weeks, resolution may occur wherein normal tissue architecture is restored. Blood clots are removed by fibrinolysis. If it is not possible to return the tissue to its original form, scarring may occur from in-filling with fibroblasts, collagen, and new endothelial cells. Generally, by this time any infection will have been overcome, although this is not always the case and may result in further immunological responses, such as granuloma formation.

Inflammation is often considered in terms of acute inflammation that includes all the events of the acute vascular and acute cellular response (1 and 2 above), and chronic inflammation that includes the events during the chronic cellular response and resolution or scarring (3 and 4).

It should be understood, however, that in addition to the occurrence of inflammatory responses in a localised fashion in tissue which is damaged, infected or subject to an autoimmune response inflammatory responses may also occur systemically, such as in the case with sepsis.

Accordingly, in light of the wide-ranging impact of inflammatory responses, there is an ongoing need to elucidate the complex mechanisms by which they function. By identifying these mechanisms there is thereby provided scope for developing means of appropriately modulating inflammatory responses.

Inhibin, activin, and follistatin are three families of polypeptides originally isolated and characterized from ovarian follicular fluid based on their modulation of follicle stimulating hormone release from pituitary cell culture. In addition to their effects on follicle stimulating hormone synthesis and secretion, inhibin and activin have other biological functions. By contrast, the physiological significance of follistatin was obscure, until it was discovered that follistatin is a binding protein to activin.

Activins, composed of two β-subunits, β_A, β_B, β_C, β_D, and/or β_E are members of the transforming growth factor (TGF)-β superfamily [Vale *et al.*, 1990, Handbook of Experimental Physiology, Vol. 95, Eds. Sporn & Roberts, Springer-Verlag, Berlin pp211-248]. Multimeric protein forms of activin include the homodimeric forms (Activin A - $\beta_A\beta_A$, Activin B - $\beta_B\beta_B$, Activin C - $\beta_C\beta_C$, Activin D - $\beta_D\beta_D$, and Activin E - $\beta_E\beta_E$) and the heterodimeric forms (for example, Activin AB - $\beta_A\beta_B$, Activin AC - $\beta_A\beta_C$, Activin AD - $\beta_A\beta_D$, or Activin AE - $\beta_A\beta_E$). The activins are multifunctional proteins. For example, Activin A, although originally identified as a regulator of follicle stimulating hormone release, is now known to exhibit the pleiotropic range of functional activities which are characteristic of most cytokines. Activins, like their related proteins, inhibins (which consist of a dimer of a structurally related but dissimilar α subunit and an activin β subunit) can bind to activin type II receptors. However, only activins are able to recruit type I receptors to form an active complex, triggering intracellular Smad signalling pathways and thereby influencing cellular function at the transcriptional level. At present, activin A, AB and B have been shown to demonstrate typical receptor-mediated agonist activity. Activin B has been reported to display less biological activity than activin A [Nakamura *et al.*, *Journal of Biological Chemistry*, 267, 16385-16389, 1992]. This may be associated with variation in the availability of specific type I receptors, differentially recruited by activin A and B [Tsuchida *et al.*, 2004 *Molecular and Cellular Endocrinology* 220, 50-65].

Follistatin functions as a biological regulator of activin. In fact, it was originally identified as an activin-binding protein. Follistatin is a monomeric protein which binds to activin with high affinity and is believed to thereafter lead to lysosomal degradation of the complexed activin. Follistatin comprises a number of post-translational and glycosylation variants. However, the two major isoforms are the full length follistatin 315, which is believed to be the predominant circulating isoform, and the 288 isoform, which has a strong affinity for heparin sulphate proteoglycans and is largely a cell membrane-associated isoform (Phillips and deKretser, 1998, *Frontiers in Neuroendocrinology* 19:287-322).

Activin affects the growth and differentiation of many cell types, stimulates the secretion of follicle-stimulating hormone from the pituitary gland and inhibits growth hormone, prolactin, and adrenocorticotropin release [Billestrup *et al.*, *Molecular Endocrinology* 1990 4:356–362; Kitaoka *et al.*, *Biochemical and Biophysical Research Communications* 1988 157:48–54; Vale *et al.*, *Nature* 1986, 321:776–779]. Activin A was first characterized for its ability to stimulate follicle stimulating hormone (FSH) from the pituitary, a capacity shared by activin B [Nakamura *et al.*, 1992, *supra;* Van Dijk *et al.*, 1995, *Annals of the New York Academy of Science* 762, 319-330]. However, activin A is now known to have many more properties besides this initial function for which it was first isolated. Both activin A and B participate in foetal development, with their respective mouse knockouts presenting distinct phenotypic anomalies. Knockouts of activin A exhibit neonatal lethal phenotypic defects [Vassalli *et al.*, 1994, *Genes and Development*, 8:414-427; Matzuk *et al.*, 1995, *Nature* 374: 354-356] but substitution of the β_A gene with β_B provides partial rescue of this phenotype [Brown *et al.*, 2000, *Nature Genetics,* 25:453-457], suggesting some overlap in the activities of activin A and B. In contrast to these observations there is evidence that activin B may have specific roles in processes such as embryonic mesoderm induction [Thomsen *et al.*, 1990, *Cell* 63:485-493] and mammary gland development· [Robinson *et al.*, 1997, *Development* 124:2701-2708]. Of particular interest is that activin B is presumed to be the activin of relevance in intrapituitary regulation of FSH, as shown by neutralization studies [Corrigan *et al.*, 1991, *Endocrinology* 128:1682-1684]. Additionally, distinct differences in expression patterns of activin A and B are evident during tissue repair [Hübner *et al.*, 1996, *Developmental Biology* 173:490-498] and in association with models of liver fibrosis [De Bleser *et al.*, 1997, *Hepatology,* 26:905-912]. Such evidence suggests that activin A and B play different roles in a range of biological and pathological processes.

Follistatin specifically binds to activin. As a result, circulating follistatin 315 neutralizes activin activity by preventing the interaction of the cytokine with its type II receptors [de Winter *et al.*, *Molecular and Cellular Endocrinology* 1996 116:105–114] and, furthermore, cell surface-bound follistatin 288 facilitates the lysosomal degradation of activin [Hashimoto *et al.*, *Journal of Biological Chemistry* 1997 272:13835–13842]. Both

follistatin and activin mRNAs show a broad tissue distribution [Meunier *et al.*, *PNAS* 1988 85:247–251; Michel *et al.*, *Biochemical and Biophysical Research Communications* 1990 173:401–407; Schneider *et al.*, *European Journal of Endocrinology* 2000 142:537–544]. Follistatin and activin are detectable in serum [Demura *et al.*, *Journal of Clinical Endocrinology and Metabolism* 1993 76:1080–1082; Demura *et al.*, *Biochemical and Biophysical Research Communications* 1992 185:1148–1154; Gilfillan *et al.*, *Clinical Endocrinology* 1994 41:453–461; Khoury *et al.*, *Journal of Clinical Endocrinology and Metabolism* 1995 80:1361–1368; Knight *et al.*, *Journal of Endocrinology* 1996 148:267–279; McFarlane *et al.*, *European Journal of Endocrinology* 1996 134:481–489; Sakai *et al.*, *Biochemical and Biophysical Research Communications* 1992 188:921–926; Sakamoto *et al.*, *European Journal of Endocrinology* 1996 135:345–351; Tilbrook *et al.*, *Journal of Endocrinology* 1996 149:55–63; Wakatsuki *et al.*, *Journal of Clinical Endocrinology and Metabolism* 1996 81:630–634], and their concentrations in serum increase with age [Wakatsuki *et al.* 1996, *supra*; Loria *et al.*, *European Journal of Endocrinology* 1998 139:487–492]. At present, however, the precise sources of follistatin and activin in serum are unknown. Current data suggest that tissue-specific balances of follistatin and activin govern the growth and differentiation of responsive cell types in an autocrine/paracrine manner [Michel *et al.*, *Acta Endocrinologica* 1993 129:525–531; Phillips, *Trends in Endocrinology and Metabolism* 2001 12:94–96].

An emerging roll for activin and follistatin in the body's innate immune response has been documented. For instance, activin and follistatin are secreted by various cell types in response to inflammatory compounds *in vitro* [Hübner *et al.*, *Experimental Cell Research* 1996 228:106–113; Jones *et al.*, *Endocrinology* 2000 141:1905–1908; Keelan *et al.*, *Placenta* 2000 21:38–43; Michel *et al.*, *Endocrinology* 1996 137:4925–4934; Phillips *et al.*, *Journal of Endocrinology* 1998 156:77–82; Yu *et al.*, *Immunology* 1996 88:368–374; Erämaa *et al.*, *Journal of Experimental Medicine* 1992 176:1449–1452; Shao *et al.*, *Cytokine* 1998 10:227–235; Mohan *et al.*, *European Journal of Endocrinology* 2001 145:505–511]. Moreover, in some examples of inflammatory processes such as wound healing, inflammatory bowel disease and rheumatoid arthritis, increased activin and/or follistatin expression has been noted [Hübner *et al.*, *Laboratory Investigation* 1997

77:311–318; Hübner *et al.*, 1996, *supra*; Yu *et al.*, *Clinical and Experimental Immunology* 1998 112:126–132]. However, since these very early and preliminary findings, the role of activin and follistatin in the context of inflammation, *per se*, has not been further elucidated, either in the context of their precise activities or in the context of the scope of the inflammatory conditions in which they function. In light of the extreme diversity in terms of the nature and extent of inflammatory responses which can occur, and the extremely pleiotropic activities of cytokines such as the various forms of activin, it is not surprising that the preliminary findings of the mid to late 1990's have not progressed to more substantial theories. In particular, activin A, activin B and follistatin are expressed by a wide variety of cell types and most organs in the body in response to a wide range of stimuli. Accordingly, their role in the context of inflammation cannot be predicted and is therefore far from clear.

In work leading up to the present invention it has been surprisingly determined that activins A functions as a crucial component of the cytokine cascade which regulates the inflammatory response. Specifically, activin A initiates the release, *in vivo*, of the pro-inflammatory cytokines and can, in fact, modulate the levels of pro-inflammatory cytokines which are released subsequently to an appropriate stimulus. Accordingly, although it has previously been observed that activin A levels are modulated during the onset and progress of an inflammatory response, until the advent of the present invention there had been no progress made in elucidating the precise role of this molecule in the context of inflammation.

It has still further been surprisingly determined that activin B levels are even more dramatically modulated in the context of an inflammatory response than are activin A levels. This is particularly surprising in light of what has been known to date in relation to the distinct roles of activins A and B. Still further, whereas immunoassays directed to the measurement of activin A have been available for use for some time, analysis of activin B has been inhibited by the absence of a specific immunoassay for this particular activin species. A very limited data set is available which suggests that circulating activin B levels alter during pregnancy or with ovarian function [Petraglia *et al.*, 1993, *Endocrine*

Journal 1:323-327; Woodruff *et al.*, 1997, *Journal of Endocrinology* 152:167-174; Vihko *et al.*, 1998, *Human Reproduction* 13:841-846; Vihko *et al.*, 2003, *Acta Obstetricia et Gynecologica Scandinavica,* 80:570-574]. Kobayashi *et al.* (2000) demonstrated that an increase in activin-β_B mRNA is associated with liver regeneration and the development of fibrosis, although the authors do not postulate whether this is linked with changes to levels of activin AB, Activin B or inhibin. A study by Rosendahl *et al.* 2001 examined a mouse model of allergen-induced airway challenge in the lung and focussed on examining associated changes in expression and distribution of TGF-β superfamily and TGF-β/activin receptors. This group reported that induced airway allergens produced only a very modest elevation of activin β_B mRNA expression over control levels. Histological examinations did not provide any information on mature activin dimer protein synthesis or distribution (either activin A or B) nor was there any evidence provided that the modest increase in activin-β_B mRNA levels was not, in fact, linked to changes in inhibin levels. Accordingly, the determination that activin B levels are in fact dramatically increased during inflammation relative to activin A levels is extremely unexpected in light of the very limited information which was available about the functioning of both the activin A and activin B molecules.

The findings of the present invention have now facilitated the development of methodology directed to modulating the inflammatory response by regulating the levels of functionally active activin A and activin B and, therefore, pro-inflammatory cytokine release. Accordingly, there are now provided both methods for the therapeutic or prophylactic treatment of conditions characterised by an unwanted or inappropriate inflammatory response and means for screening for regulators of pro-inflammatory cytokine release such as activin A and activin B mimetics, agonists or antagonists.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The subject specification contains nucleotide sequence information prepared using the programme PatentIn Version 3.1, presented herein after the bibliography. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (eg. <210>1, <210>2, etc). The length, type of sequence (DNA, etc) and source organism for each nucleotide sequence is indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are identified by the indicator SEQ ID NO: followed by the sequence identifier (eg. SEQ ID NO:1, SEQ ID NO:2, etc.). The sequence identifier referred to in the specification correlates to the information provided in numeric indicator field <400> in the sequence listing, which is followed by the sequence identifier (eg. <400>1, <400>2, etc). That is SEQ ID NO:1 as detailed in the specification correlates to the sequence indicated as <400>1 in the sequence listing.

One aspect of the present invention is directed to a method of modulating the inflammatory response in a mammal, said method comprising modulating the functional activity of activin wherein upregulating activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating activin to a functionally ineffective level in said mammal inhibits or retards the pro-inflammatory mediator cascade.

Another aspect of the present invention is directed to a method of modulating the inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal

induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level in said mammal inhibits or retards the pro-inflammatory mediator cascade.

In still another aspect the present invention is directed to a method of modulating a local inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level in said mammal inhibits or retards the local pro-inflammatory mediator cascade.

In yet another aspect the present invention is directed to a method of modulating a systemic inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level in said mammal inhibits or retards the systemic pro-inflammatory mediator cascade.

In still yet another aspect the present invention is directed to a method of modulating the inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level in said mammal inhibits or retards the pro-inflammatory cytokine cascade.

In yet still another aspect the present invention is directed to a method of modulating a local inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising

a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level in said mammal inhibits or retards the local pro-inflammatory cytokine cascade.

In a further aspect the present invention is directed to a method of modulating a systemic inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level in said mammal inhibits or retards the systemic pro-inflammatory cytokine cascade.

In another further aspect the present invention is directed to a method of down-regulating the inflammatory response in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to induce a functionally ineffective level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in said mammal.

In yet another further aspect there is provided a method of up-regulating the inflammatory response in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to induce a functionally effective level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in said mammal.

Still another further aspect of the present invention contemplates a method of therapeutically and/or prophylactically treating a condition, or a predisposition to the development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal, said method comprising modulating the level of activin in said mammal where up-regulating activin to a functionally effective level up-regulates the pro-inflammatory mediator cascade and down-regulating activin to a

functionally ineffective level inhibits or retards the pro-inflammatory mediator cascade.

Yet still another further aspect of the present invention contemplates a method of therapeutically and/or prophylactically treating a condition or a predisposition to the development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal, said method comprising modulating the level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level inhibits or retards the pro-inflammatory cytokine cascade.

In still yet another further aspect there is provided a method of therapeutically and/or prophylactically treating a condition, or a predisposition to the development of a condition, characterised by an unwanted acute inflammatory response in a mammal, said method comprising down-regulating the level activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein downregulating said activin to a functionally ineffective level inhibits or retards the pro-inflammatory cytokine cascade.

In another aspect there is provided a method of therapeutically and/or prophylactically treating a condition, or a predisposition to the development of a condition, characterised by an inadequate inflammatory response in a mammal, said method comprising modulating the level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally effective level up-regulates the pro-inflammatory cytokine cascade.

Still another aspect of the present invention relates to the use of an agent capable of modulating the functionally effective level of activin in the manufacture of a medicament for the therapeutic and/or prophylactic treatment of a condition, or a predisposition to the

development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal wherein up-regulating activin to a functionally effective level up-regulates the pro-inflammatory mediator cascade and down-regulating activin to a functionally ineffective level inhibits or retards the pro-inflammatory mediator cascade.

Yet another aspect of the present invention relates to the use of an agent capable of modulating the functionally effective level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in the manufacture of a medicament for the therapeutic and/or prophylactic treatment of a condition, or a predisposition to the development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal wherein up-regulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level inhibits or retards the pro-inflammatory cytokine cascade.

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows activin A release following an inflammatory challenge, in the form of lipopolysaccharide (LPS), in mice.

Figure 1B shows follistatin release in response to LPS.

Figure 1C shows TNFα release in response to LPS.

Figure 1D shows IL-6 release in response to LPS.

Figure 1E shows IL-1β release in response to LPS.

Figure 2A shows activin A release following an injection of LPS in mice that received an injection of recombinant human follistatin-288 (rhfollistatin-288) 30 minutes prior to LPS.

Figure 2B shows release of follistatin in mice following administration of rhfollistatin-288 30 minutes prior to LPS.

Figure 2C shows the level of TNFα released in mice following administration of rhfollistatin-288 30 minutes prior to LPS.

Figure 2D shows the level of interleukin-6 released following injection of rhfollistatin-288 followed by an injection of LPS.

Figure 2E shows the level of IL-1β released following injection of follistatin followed by LPS.

Figure 3: Data showing activin A expression in (A) bronchial epithelium and inflammatory infiltrate, (B) diffuse expression on submucosal smooth muscle and vascular structures (arrows) and (C) expression on bronchial epithelium and discrete inflammatory

cells (arrows). A and B, asthma; C, cystic fibrosis. Immunoperoxidase, original magnification x 400.

Figure 4: Data showing kinetics of activin A expression and lung inflammation in our OVA murine model. (A) Activin concentration in BALF as measured by ELISA, (B) absolute eosinophil numbers in BAL, and (C) frequency of IL-4–producing mediastinal lymph node cells as measured by ELISPOT. Mean ± SEM, n = 5 mice per group per time-point.

Figure 5: Data showing activin A expression in saline control (A) and OVA sensitised mice after 4 challenges (B), and 10 days after the 4th challenge (C). Arrows indicate loss of activin A expression in hypertrophied bronchial epithelium (B), and patchy expression at day 17 (C).

Figure 6: Quantitative mRNA levels for activin β_A (upper panel) and βB (lower panel) subunits in livers of mice challenged with a single intraperitoneal injection of LPS. Mice were either treated with LPS alone (no follistatin pretreatment, solid circles) or 1 μg of human recombinant follistatin 288 thirty minutes before LPS (follistatin pretreatment, open circles). Data are represented as mean ± SEM at each timepoint assessed relative to LPS, with expression levels expressed relative to the expression of the housekeeping gene, GADPH. All time 0 data were normalized to a value of 1 and data at subsequent time points were expressed relative to that time point.

Figure 7: Quantitative mRNA levels for activin β_A (upper panel) and βB (lower panel) subunits in livers of mice challenged with a single intraperitoneal injection of CCl_4. Data are represented as mean ± SEM at each timepoint assessed relative to CCl_4, with expression levels expressed relative to the expression of the housekeeping gene, GADPH. All time 0 data were normalized to a value of 1 and data at subsequent time points were expressed relative to that time point.

Figure 8: Immunolocalization of the activin β_A subunit in livers of mice at various timepoints following LPS treatment. The activin β_A subunit was localized to hepatocytes in untreated animals (t=0 hr) but predominantly around the central hepatic veins. Immunolocalization appeared diminished at 5 hours following LPS challenge, but returned to pre-treatment localization patterns by 12 hours (X 50).

Figure 9: Immunolocalization of the activin βB subunit in livers of mice at various timepoints following LPS treatment. The activin βB subunit was localized to hepatocytes in untreated animals (t=0 hr), in areas surrounding portal tracts but not central veins. Immunolocalization appeared diminished at 5 hours following LPS challenge, but returned to pre-treatment localization patterns by 12 hours. Note also the loss of localization in peripheral hepatocytes (asterisks) (X 50).

Figure 10: Immunolocalization of the activin β_A subunit (panels a and b) and activin βB subunit (panels c and d) in livers of mice at 0 or 36 hours following CCl_4 challenge. As for LPS treatment, the activin βB subunit was localized to areas surrounding the portal tract but not central veins whereas the activin β_A subunit predominantly localized to hepatocytes surrounding central veins. Note also the localization of activin β_A subunit at the 36 hour timepoint in areas of hepatocyte apoptosis/necrosis, while localization for the activin β_B subunit is absent from these areas (asterisks) (X50).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the surprising determination that the role of activins A and B in the inflammatory response occur in the context of these molecules being modulators of pro-inflammatory cytokine release. Specifically, activin A has been found to initiate the onset of the pro-inflammatory cytokine cascade. Similarly, but still more surprisingly, activin molecules comprising the β_B subunit have now also been found to regulate the very early stages of the inflammatory response, despite otherwise exhibiting significant functional distinctiveness to activin A. Most surprisingly, however, this molecule exhibits significantly higher levels of expression than activin A at this time. Accordingly, these findings have now facilitated the rational design of means for modulating the inflammatory response and, in particular, for therapeutically or prophylactically treating conditions which are characterised by an inappropriate inflammatory response. Further, there is facilitated the identification and/or design of agents which specifically interact with or mimic activin A or an activin molecule comprising a β_B subunit to modulate its functionality and thereby the onset or progression of an inflammatory response.

Accordingly, one aspect of the present invention is directed to a method of modulating the inflammatory response in a mammal, said method comprising modulating the functional activity of activin wherein upregulating activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating activin to a functionally ineffective level in said mammal inhibits or retards the pro-inflammatory mediator cascade.

More particularly, the present invention is directed to a method of modulating the inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising the β_B subunit wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the pro-inflammatory mediator cascade and downregulating said activin to a functionally ineffective level in said mammal inhibits or

retards the pro-inflammatory mediator cascade.

Without limiting the present invention to any one theory or mode of action, the inflammatory response is a complex response characterised by a series of physiological and/or immunological events which are induced to occur by the release of a cytokine cascade in response to any one of a variety of stimuli including, but not limited to, tissue injury, infection, an immune response (such as to a pathogen or an innocuous agent – as occurs with allergies), or disease (such as tumour formation or an autoimmune response).

The physiological events which characterise inflammation include:

(i) vasodilation
(ii) increased vascular permeability
(iii) cellular infiltration
(iv) changes to the biosynthetic, metabolic and catabolic profiles of affected organs
(v) activation of the cells of the immune system.

It should be understood that reference to an "inflammatory response" is a reference to any one or more of the physiological and/or immunological events or phases that are induced to occur in the context of inflammation and, specifically, in response to the signals generated by the cytokine cascade which directs the inflammatory response. For example IL-1, TNFα and IL-6 are well known for their functions as pro-inflammatory mediators. It should also be understood that an inflammatory response within the context of the present invention essentially includes a reference to a partial response, such as a response which has only just commenced, or to any specific phase or event of a response (such as the phases and events detailed in points (i)-(v), above, or any other effect related to inflammation including, but not limited to, the production of acute phase proteins – including complement components, fever and a systemic immune response). Further, it should also be understood that depending on any given set of specific circumstances, the end point of an inflammatory response may vary. For example, in some situations there may only occur an acute vascular response. To the extent that "acute" inflammation

occurs, this is generally understood to include the events of both an acute vascular response and an acute cellular response. Some inflammatory responses will resolve at the acute stage while others may progress to become chronic cellular responses.

Without limiting the present invention to any one theory or mode of action, in certain circumstances the acute process, characterized by neutrophil infiltration and oedema, gives way to a predominance of mononuclear phagocytes and lymphocytes. This is thought to occur to some degree with the normal healing process but becomes exaggerated and chronic when there is ineffective elimination of foreign materials as in certain infections (e.g. tuberculosis) or following introduction of foreign bodies (e.g. asbestos) or deposition of crystals (e.g. urate crystals). Chronic inflammation is often associated with fusion of mononuclear cells to form multinucleated gigant cells, which eventually become a granuloma. Chronic inflammation is also seen under conditions of delayed hypersensitivity. The subject inflammatory response may be systemic or localised. Examples of systemic inflammatory responses include those which fall within the scope of systemic inflammatory response syndrome such as septic shock, toxic shock or septicaemia.

Examples of localised inflammatory responses include those which occur in the context of airway inflammation (for example, asthma, interstitial lung disease, cystic fibrosis, lung transplantation, bronchiolitis obliterans, emphysema, obstructive pulmonary disease, asbestosis, obstructive sleep apnoea, hypoxia or pulmonary hypertension), rheumatoid arthritis, inflammatory bowel disease, pancreatitis, atherosclerosis, meningitis, appendicitis, angiogenesis, psoriasis, neural protection, renal tubular necrosis, allergic responses and wound healing (for example, pursuant to surgery, burns or other tissue injury). It should be understood, however, that some localised inflammatory responses can become systemic, for example as can occur when the onset of septic shock occurs as a complication of severe burns or abdominal wounds. In another example, septicaemia can result from the transition of a more localised bacterial infection to a circulatory infection.

Accordingly, in one preferred embodiment the present invention is directed to a method of

modulating a local inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising the β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or upregulates the local pro-inflammatory mediator cascade and down-regulating said activin to a functionally ineffective level in said mammal inhibits or retards the local pro-inflammatory mediator cascade.

More preferably, said local inflammatory response is acute.

In another preferred embodiment the present invention is directed to a method of modulating a systemic inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising the β_B subunit, wherein upregulating said activin to a functionally effective level in said mammal induces, maintains or up-regulates the systemic pro-inflammatory mediator cascade and down-regulating said activin to a functionally ineffective level in said mammal inhibits or retards the systemic pro-inflammatory mediator cascade.

More preferably, said systemic inflammatory response is acute.

In accordance with these preferred aspects of the present invention, said acute inflammatory response is preferably down-regulated and occurs in the context of, or is otherwise associated with, septic shock, septicaemia, airway inflammation, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, or wound healing or traumatic injury such as occurs with surgery and burns.

Preferably, said airway inflammation occurs in the context of asthma, intersticial lung disease, cystic fibrosis, lung transplantation, bronchiolitis obliterans, emphysema, obstructive pulmonary disease, asbestosis, obstructive sleep apnoea, hypoxia or pulmonary

hypertension.

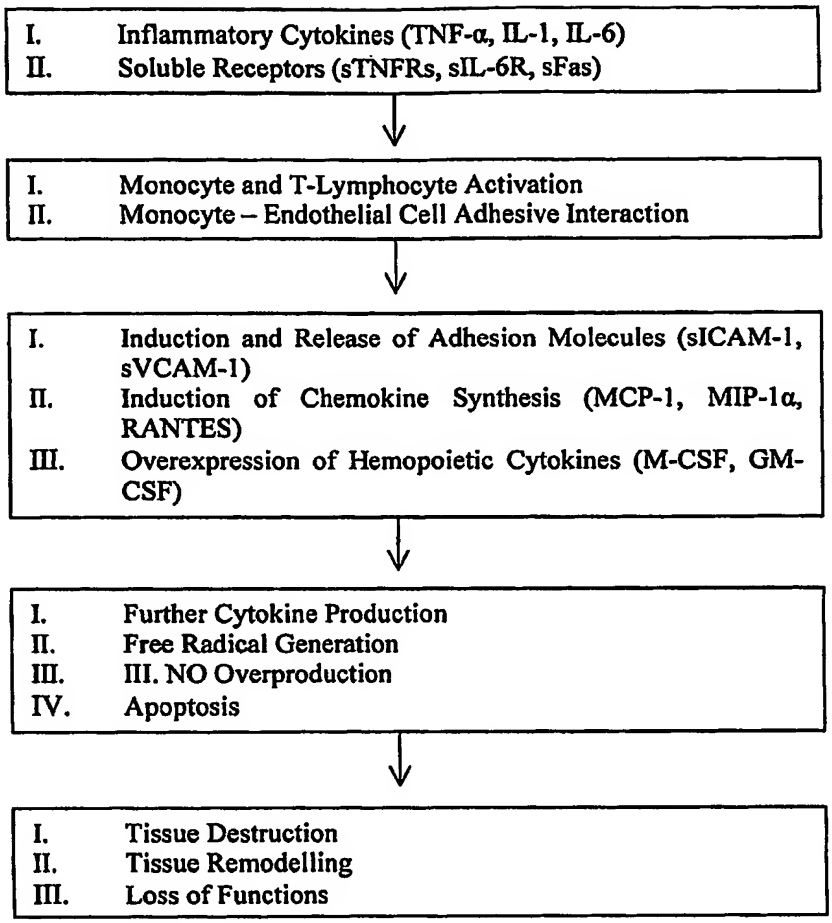
Preferably, said acute systemic inflammatory response occurs in the context of systemic inflammatory response syndrome and even more particularly sepsis, septicaemia, toxic shock, septic shock, tissue trauma, meningitis or appendicitis.

Reference to "activin A" should be understood as a reference to all forms of activin A and to fragments, derivatives, mutants or variants thereof. Activin A is a dimeric protein which comprises two activin β_A subunits. It should also be understood to include reference to a dimer comprising any isoforms which may arise from alternative splicing of activin β_A mRNA or mutant or polymorphic forms of activin β_A. Reference to "activin A" should be understood to include reference to all forms of these molecules including all precursor, proprotein or intermediate forms thereof. Reference to activin A should also be understood to extend to any activin A protein, whether existing as a dimer, multimer or fusion protein.

Reference to "an activin molecule comprising a β_B subunit" should be understood as a reference to a monomeric or multimeric molecule, preferably a dimer, which comprises at least one activin β_B subunit. Reference to "activin β_B" should be understood as a reference to all forms of activin β_B and to fragments, derivatives, mutants or variants thereof. "Activin β_B subunit" is also interchangeably referred to as "activin β_B". It should be understood to include reference to any isoforms which may arise from alternative splicing of activin β_B mRNA or mutant or polymorphic forms of activin β_B. Reference to "activin β_B" is not intended to be limiting and should be read as including reference to all forms of activin β_B including any protein encoded by the activin β_B subunit gene, any subunit polypeptide such as precursor forms which may be generated, and any β_B protein, whether existing as a monomer, multimer or fusion protein. Multimeric protein forms of activin β_B include for example the homodimeric activin B (β_B-β_B) or the heterodimeric activin AB (β_A-β_B), activin BC (β_B-β_C), activin BD (β_B-β_D) or activin BE (β_B-β_E) proteins. Preferably, said activin molecule is activin B.

Reference to "modulating" should be understood as a reference to up-regulating or down-regulating the subject inflammatory response. Reference to "down-regulating" an inflammatory response should therefore be understood as a reference to preventing, reducing (eg. slowing) or otherwise inhibiting one or more aspects of an inflammatory response while reference to "up-regulating" should be understood to have the converse meaning. In the context of the present invention, modulation of the inflammatory response is achieved via up-regulation or down-regulation of the pro-inflammatory cytokine cascade. Although the preferred method is to down-regulate the inflammatory response in the context of conditions characterised by an unwanted inflammatory response, such as airway inflammation, sepsis, septicaemia, meningitis, rheumatoid arthritis or tissue trauma, the present invention nevertheless extends to up-regulating the inflammatory response in circumstances where it is desired that an inflammatory response occur. This may occur, for example, in situations where an inflammatory response is required to provide adjuvant-like activity. This may be particularly useful in the context of anti-tumour therapy. In still another example, the upregulation of host defence mechanisms may be desired.

Without limiting the present invention to any one theory or mode of action, inflammation is a complex biological process which involves the interaction, in a cascade fashion, of numerous soluble mediators. Briefly, the cascade of cytokines and other inflammatory mediators which act to induce an inflammatory response can be schematically depicted as follows:



Accordingly, reference to "pro-inflammatory mediator cascade" should be understood as a reference to the sequential interaction of soluble molecules which characterise the onset and progression of an inflammatory response. In particular, the onset of an inflammatory mediator cascade is characterised by the sequential up-regulation of expression of TNF-α, IL-1 and IL-6. However, the entire inflammatory process is nevertheless characterised by sequential changes in the levels of various cytokines (the term "cytokines" should be broadly understood to include reference to the interleukins, chemokines, monokines, colony stimulating factors and other such protein hormones). Despite prior observations that levels of activin are modulated in mammals experiencing an inflammatory response, the precise role of activin in this context was not understood. To this end, the pro-

inflammatory cytokines are still generally understood to be constituted by TNF-α, IL-1 and IL-6. Still further, and without limiting the present invention in any way, TNF-α is secreted in response to various pro-inflammatory stimuli and exerts a wide variety of effects. At low concentrations, it acts as a paracrine and autocrine molecule, upregulating vascular adhesion molecules, activating neutrophils, and stimulating monocytes to secrete Interleukin 1, 6 and more TNF-α. At higher concentrations, TNF-α enters the serum and becomes an endocrine hormone. Here, it acts as a pyrogen, stimulates further cytokine liberation from mononuclear cells, activates the coagulation system, and suppresses bone marrow stem cell maturation. At even higher concentrations, TNF-α has many deleterious effects, including hypotension (probably through induction of nitric oxide [NO] synthesis) and induction of disseminated intravascular coagulation (DIC).

IL-1 is also produced by activated mononuclear cells in response to pro-inflammatory stimuli. IL-1 has two forms: IL-1α and IL-1β. IL-1α is active as its 33kD molecule; IL-1β needs to be cleaved further to a 17kD biologically active peptide. The endocrine effects of high doses of IL-1β are similar to TNF-α, causing fever, DIC, and metabolic wasting. Activated monocytes also produce IL-6 in response to IL-1 and TNF-α stimulation. IL-6 then acts on hepatocytes and B cells to propagate the inflammatory process. Under IL-6 stimulation, hepatocytes secrete increased levels of acute phase reactants, such as fibrinogen. IL-6 also acts as a B-cell growth factor, thereby promoting antibody formation and release.

In terms of modulating the inflammatory response (particularly down-regulating the response), modulation of the cytokine cascade has been a primary focus. Attempts have been made to alter the pro-inflammatory cytokine cascade to block a particular inflammatory molecule, thereby theoretically altering the cascade and potentially benefiting the patient. TNF-α and IL-1 are two such molecules targeted for modulation. Therapies with anti-TNF-α antibody and IL-1 receptor antagonist have been tested. However, to date targeting one specific cytokine or inflammatory mediator for immunotherapy has not generally proven to be a useful proposition for treatment. In this regard, it has generally been regarded that since any cytokine or mediator is only one

component of the cascade, neutralising one agent is unlikely to down-regulate the entire cascade. It is for these reasons that the present findings are so surprising. First, it has been determined that the pro-inflammatory mediator cascade, from its earliest stages, involves modulation in the level of activin A expression. Specifically, activin A levels are increased shortly after the inflammatory stimulus occurs and prior to TNF-α, IL-1 and IL-6 expression. Accordingly, activin A appears to be involved in the initiation of the pro-inflammatory cytokine cascade. Still further, it has been determined that down-regulating activin A functionality can, in fact, achieve the favourable outcome of down-regulating the inflammatory response. A role for activin B during the early stages of the pro-inflammatory cytokine cascade has also been surprisingly elucidated. Still more surprisingly, however, has been the determination that the levels of activin B which are observed during this phase of an inflammatory response are significantly higher than the corresponding activin A levels.

Accordingly, in a preferred embodiment the present invention is directed to a method of modulating the inflammatory response in a mammal, said method comprising modulating the functional activity of activin, which activin is activin A or an activin molecule comprising the β_B subunit, wherein up-regulating said activin to a functionally effective level in said mammal induces, maintains or up-regulates the pro-inflammatory cytokine cascade and down-regulating said activin to a functionally ineffective level in said mammal inhibits or retards the pro-inflammatory cytokine cascade.

Preferably, said activin is activin A and/or activin B.

In one embodiment the present invention is directed to a method of modulating a local inflammatory response in a mammal, said method comprising modulating the functional activity of activin A and/or activin B wherein up-regulating activin to a functionally effective level in said mammal induces, maintains or up-regulates the local pro-inflammatory cytokine cascade and down-regulating activin A and/or activin B to a functionally ineffective level in said mammal inhibits or retards the local pro-inflammatory cytokine cascade.

Preferably, said local inflammatory response is acute.

In another preferred embodiment the present invention is directed to a method of modulating a systemic inflammatory response in a mammal, said method comprising modulating the functional activity of activin A and/or activin B wherein up-regulating activin to a functionally effective level in said mammal induces, maintains or up-regulates the pro-inflammatory cytokine cascade and down-regulating activin A and/or activin B to a functionally ineffective level in said mammal inhibits or retards the systemic pro-inflammatory cytokine cascade.

Preferably, said systemic inflammatory is acute.

In accordance with these preferred aspects of the present invention, said acute inflammatory response is preferably down-regulated and occurs in the context of, or is otherwise associated with, septic shock, septicaemia, airway inflammation, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, or wound healing or traumatic injury such as occurs with surgery and burns.

Preferably, said airway inflammation occurs in the context of asthma, intersticial lung disease, cystic fibrosis, lung transplantation, bronchiolitis obliterans, emphysema, obstructive pulmonary disease, asbestosis, obstructive sleep apnoea, hypoxia or pulmonary hypertension.

Preferably, said acute systemic inflammatory response occurs in the context of systemic inflammatory response syndrome and even more particularly sepsis, septicaemia, toxic shock, septic shock, tissue trauma, meningitis or appendicitis.

In accordance with these preferred embodiments, said pro-inflammatory cytokine cascade corresponds to the expression of TNF-α, IL-1 and/or IL-6.

It should be understood that in terms of modulating the pro-inflammatory cytokine cascade, this may be achieved either by modulating the actual levels of these cytokines or by modulating their functionality. For example, and without limiting the present invention to any one theory or mode of action, it has been shown that administration of follistatin (this molecule functioning as an activin antagonist) prior to the LPS challenge of a mammal nevertheless results in the expression of an activin A peak at a concentration which is the same as is normally observed to occur during inflammation. However, due to binding of the follistatin to the activin A, thereby blocking the functionality of activin A, the concentration of TNF-α which is expressed drops by 50%. Interestingly, the concentration of expressed IL-6 is observed to increase 6 fold at a significantly earlier time point. In total, these changes in the pro-inflammatory cytokine profile nevertheless result in a decrease in the observed inflammatory response. These findings are based on protein measurement and therefore indicate secretion and/or release of mature activin A dimeric protein. Accordingly, follistatin pre-treatment does not appear to affect this process. However, where activin βA and/or βB mRNA are measured, follistatin pre-treatment does in fact ameliorate the synthesis mechanisms of both activin subunit genes. With respect to the activin βA subunit, the inhibition of the mRNA is not reflected in protein release. The same mechanism is postulated to apply in the context of activin βB. Accordingly, still without limiting the present invention in any way, there occurs a rapid release of essentially pre-stored protein and then a follistatin-regulated synthesis pathway that is separate from this release mechanism. Of most significance, however, is the unexpected determination that there is observed a small increase in activin βA mRNA following inflammatory challenge but a massive increase in activin βB mRNA by the same stimulation.

Reference herein to attaining either a "functionally effective level" or "functionally ineffective level" of activin should be understood as a reference to attaining that level of activin at which modulation of the inflammatory response can be achieved, whether that be up-regulation or down-regulation. In this regard, it is within the skill of the person of skill

in the art to determine, utilising routine procedures, the threshold level of activin expression above which or below which inflammation is modulated.

It should be understood that reference to an "effective level" means the level necessary to at least partly attain the desired response. The amount may vary depending on the health and physical condition of the cellular population and/or individual being treated, the taxonomic group of the cellular population and/or individual being treated, the degree of up or down-regulation which is desired, the formulation of the composition which is utilised, the assessment of the medical situation and other relevant factors. Accordingly, it is expected that this level may vary between individual situations, thereby falling in a broad range, which can be determined through routine trials.

Modulating activin levels may be achieved by any suitable means including, but not limited to:

(i) Modulating absolute levels of activin such that either more or less activin is present in the cellular environment.

(ii) Agonising or antagonising activin protein functional activity such that the functional effectiveness of activin is either increased or decreased. For example, increasing the half life of activin may achieve an increase in the functionally effective level of activin without actually necessitating an increase in the absolute concentration of activin. Similarly, the partial antagonism of activin may act to reduce, although not necessarily eliminate, the functional effectiveness of said activin.

Accordingly, this may provide a means of down-regulating activin functioning without necessarily down-regulating absolute concentrations of activin.

In terms of achieving the up or down-regulation of activin, means for achieving this objective would be well known to the person of skill in the art and include, but are not limited to:

(i) Introducing into a cell a nucleic acid molecule encoding activin or in order to up-regulate the capacity of said cell to express activin.

(ii) Introducing into a cell a proteinaceous or non-proteinaceous molecule which modulates transcriptional and/or translational regulation of a gene, wherein this gene may be the activin gene or functional portion thereof or some other gene or gene region (eg. promoter region) which directly or indirectly modulates the expression of the activin gene.

(iii) Introducing into a cell the activin expression product (this should be understood to include the use of activin homologues).

(iv) Introducing a proteinaceous or non-proteinaceous molecule which functions as an antagonist to the activin expression product.

(v) Introducing a proteinaceous or non-proteinaceous molecule which functions as an agonist of the activin expression product.

The proteinaceous molecules described above may be derived from any suitable source such as natural, recombinant or synthetic sources and includes fusion proteins or molecules which have been identified following, for example, natural product screening. The reference to non-proteinaceous molecules may be, for example, a reference to a nucleic acid molecule or it may be a molecule derived from natural sources, such as for example natural product screening, or may be a chemically synthesised molecule. The present invention contemplates analogues of the activin expression product or small molecules capable of acting as agonists or antagonists. Chemical agonists may not necessarily be derived from the activin expression product but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to meet certain physiochemical properties. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing activin from carrying out its normal biological function.

Antagonists include monoclonal antibodies and antisense nucleic acids which prevent transcription or translation of activin genes or mRNA in mammalian cells. Modulation of expression may also be achieved utilising antigens, RNA, ribosomes, DNAzymes, aptamers, antibodies or molecules suitable for use in cosuppression. . Suitable antisense oligonucleotide sequences (single stranded DNA fragments) of activin may be created or identified by their ability to suppress the expression of activin. The production of antisense oligonucleotides for a given protein is described in, for example, Stein and Cohen, 1988 (Cancer Res 48:2659-68) and van der Krol et al., 1988 (Biotechniques 6:958-976) .

The proteinaceous and non-proteinaceous molecules referred to in points (i)-(v), above, are herein collectively referred to as "modulatory agents". Preferably, said modulatory agent is follistatin, to the extent that it is sought to decrease activin activity.

In this regard, reference to "follistatin" should be read as including reference to all forms of follistatin and to fragments, derivatives, mutants or variants thereof including, by way of example, the three protein cores and six molecular weight forms which have been identified as arising from the alternatively spliced mRNAs FS315 and FS288. Accordingly, it should also be understood to include reference to any isoforms which may arise from alternative splicing of follistatin mRNA or mutant or polymorphic forms of follistatin. It should still further be understood to extend to any protein encoded by the follistatin gene, any subunit polypeptide, such as precursor forms which may be generated, and any follistatin protein, whether existing as a monomer, multimer or fusion protein.

Screening for the modulatory agents hereinbefore defined can be achieved by any one of several suitable methods including, but in no way limited to, contacting a cell comprising the activin gene or functional equivalent or derivative thereof with an agent and screening for the modulation of activin protein production or functional activity, modulation of the expression of a nucleic acid molecule encoding activin or modulation of the activity or expression of a downstream activin cellular target. Detecting such modulation can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift

assays and/or the readout of reporters of activin activity such as luciferases, CAT and the like.

It should be understood that the activin gene or functional equivalent or derivative thereof may be naturally occurring in the cell which is the subject of testing or it may have been transfected into a host cell for the purpose of testing. Further, the naturally occurring or transfected gene may be constitutively expressed - thereby providing a model useful for, inter alia, screening for agents which down regulate activin activity, at either the nucleic acid or expression product levels, or the gene may require activation - thereby providing a model useful for, inter alia, screening for agents which up-regulate activin expression. Further, to the extent that an activin nucleic acid molecule is transfected into a cell, that molecule may comprise the entire activin gene or it may merely comprise a portion of the gene such as the portion which regulates expression of the activin product. For example, the activin promoter region may be transfected into the cell which is the subject of testing. In this regard, where only the promoter is utilised, detecting modulation of the activity of the promoter can be achieved, for example, by ligating the promoter to a reporter gene. For example, the promoter may be ligated to luciferase or a CAT reporter, the modulation of expression of which gene can be detected via modulation of fluorescence intensity or CAT reporter activity, respectively. In another example, the subject of detection could be a downstream activin regulatory target, rather than activin itself. Yet another example includes activin binding sites ligated to a minimal reporter. Modulation of activin activity can be detected by screening for the modulation of pro-inflammatory cytokine release. This is an example of an indirect system where modulation of activin expression, per se, is not the subject of detection. Rather, modulation of the down-stream activity which activin regulates is monitored.

These methods provide a mechanism for performing high throughput screening of putative modulatory agents such as the proteinaceous or non-proteinaceous agents comprising synthetic, combinatorial, chemical and natural libraries. These methods will also facilitate the detection of agents which bind either the activin nucleic acid molecule or expression product itself or which modulate the expression of an upstream molecule, which upstream

molecule subsequently modulates activin expression or expression product activity. Accordingly, these methods provide a mechanism of detecting agents which either directly or indirectly modulate activin expression and/or activity.

The agents which are utilised in accordance with the method of the present invention may take any suitable form. For example, proteinaceous agents may be glycosylated or unglycosylated, phosphorylated or dephosphorylated to various degrees and/or may contain a range of other molecules used, linked, bound or otherwise associated with the proteins such as amino acids, lipid, carbohydrates or other peptides, polypeptides or proteins. Similarly, the subject non-proteinaceous molecules may also take any suitable form. Both the proteinaceous and non-proteinaceous agents herein described may be linked, bound otherwise associated with any other proteinaceous or non-proteinaceous molecules. For example, in one embodiment of the present invention said agent is associated with a molecule which permits its targeting to a localised region.

The subject proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate the expression of activin or the activity of the activin expression product. Said molecule acts directly if it associates with the activin nucleic acid molecule or expression product to modulate expression or activity, respectively. Said molecule acts indirectly if it associates with a molecule other than the activin nucleic acid molecule or expression product which other molecule either directly or indirectly modulates the expression or activity of the activin nucleic acid molecule or expression product, respectively. Accordingly, the method of the present invention encompasses the regulation of activin nucleic acid molecule expression or expression product activity via the induction of a cascade of regulatory steps.

The term "expression" refers to the transcription and translation of a nucleic acid molecule. Reference to "expression product" is a reference to the product produced from the transcription and translation of a nucleic acid molecule. Reference to "modulation" should be understood as a reference to up-regulation or down-regulation.

"Derivatives" of the molecules herein described (for example activin A, activin B, follistatin or other proteinaceous or non-proteinaceous agents) include fragments, parts, portions or variants from either natural or non-natural sources. Non-natural sources include, for example, recombinant or synthetic sources. By "recombinant sources" is meant that the cellular source from which the subject molecule is harvested has been genetically altered. This may occur, for example, in order to increase or otherwise enhance the rate and volume of production by that particular cellular source. Parts or fragments include, for example, active regions of the molecule. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in a sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins, as detailed above.

Derivatives also include fragments having particular epitopes or parts of the entire protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, follistatin, or derivative thereof may be fused to a molecule to facilitate its localisation to a particular site. Analogues of the molecules contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues.

Derivatives of nucleic acid sequences which may be utilised in accordance with the method of the present invention may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid

molecules. The derivatives of the nucleic acid molecules utilised in the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules. Derivatives of nucleic acid sequences also include degenerate variants.

A "variant" or "mutant" of activin or follistatin should be understood to mean molecules which exhibit at least some of the functional activity of the form of activin or follistatin of which it is a variant or mutant. A variation or mutation may take any form and may be naturally or non-naturally occurring.

A "homologue" is meant that the molecule is derived from a species other than that which is being treated in accordance with the method of the present invention. This may occur, for example, where it is determined that a species other than that which is being treated produces a form of activin or follistatin, for example, which exhibits similar and suitable functional characteristics to that of the activin or follistatin which is naturally produced by the subject undergoing treatment.

Chemical and functional equivalents should be understood as molecules exhibiting any one or more of the functional activities of the subject molecule, which functional equivalents may be derived from any source such as being chemically synthesised or identified via screening processes such as natural product screening. For example chemical or functional equivalents can be designed and/or identified utilising well known methods such as combinatorial chemistry or high throughput screening of recombinant libraries or following natural product screening. Antagonistic agents can also be screened for utilising such methods.

For example, libraries containing small organic molecules may be screened, wherein organic molecules having a large number of specific parent group substitutions are used. A general synthetic scheme may follow published methods (eg., Bunin BA, et al. (1994) Proc. Natl. Acad. Sci. USA, 91:4708-4712; DeWitt SH, et al. (1993) Proc. Natl. Acad. Sci. USA, 90:6909-6913). Briefly, at each successive synthetic step, one of a plurality of

different selected substituents is added to each of a selected subset of tubes in an array, with the selection of tube subsets being such as to generate all possible permutation of the different substituents employed in producing the library. One suitable permutation strategy is outlined in US. Patent No. 5,763,263.

There is currently widespread interest in using combinational libraries of random organic molecules to search for biologically active compounds (see for example U.S. Patent No. 5,763,263). Ligands discovered by screening libraries of this type may be useful in mimicking or blocking natural ligands or interfering with the naturally occurring ligands of a biological target. In the present context, for example, they may be used as a starting point for developing activin analogues which exhibit properties such as more potent pharmacological effects. Activin or a functional part thereof may according to the present invention be used in combination libraries formed by various solid-phase or solution-phase synthetic methods (see for example U.S. Patent No. 5,763,263 and references cited therein). By use of techniques, such as that disclosed in U.S. Patent No. 5,753,187, millions of new chemical and/or biological compounds may be routinely screened in less than a few weeks. Of the large number of compounds identified, only those exhibiting appropriate biological activity are further analysed.

With respect to high throughput library screening methods, oligomeric or small-molecule library compounds capable of interacting specifically with a selected biological agent, such as a biomolecule, a macromolecule complex, or cell, are screened utilising a combinational library device which is easily chosen by the person of skill in the art from the range of well-known methods, such as those described above. In such a method, each member of the library is screened for its ability to interact specifically with the selected agent. In practising the method, a biological agent is drawn into compound-containing tubes and allowed to interact with the individual library compound in each tube. The interaction is designed to produce a detectable signal that can be used to monitor the presence of the desired interaction. Preferably, the biological agent is present in an aqueous solution and further conditions are adapted depending on the desired interaction. Detection may be

performed for example by any well-known functional or non-functional based method for the detection of substances.

In addition to screening for molecules which mimic the activity of activin one may identify and utilise molecules which function agonistically or antagonistically to activin in order to up or down-regulate the functional activity of activin in relation to modulating cellular growth. The use of such molecules is described in more detail below. To the extent that the subject molecule is proteinaceous, it may be derived, for example, from natural or recombinant sources including fusion proteins or following, for example, the screening methods described above. The non-proteinaceous molecule may be, for example, a chemical or synthetic molecule which has also been identified or generated in accordance with the methodology identified above. Accordingly, the present invention contemplates the use of chemical analogues of activin capable of acting as agonists or antagonists. Chemical agonists may not necessarily be derived from activin but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of activin. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing activin from carrying out its normal biological functions. Antagonists include monoclonal antibodies specific for activin or parts of activin. Preferably, said antagonist is follistatin.

Analogues of activin or of activin agonistic or antagonistic agents contemplated herein include, but are not limited to, modifications to side chains, incorporating unnatural amino acids and/or derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the analogues. The specific form which such modifications can take will depend on whether the subject molecule is proteinaceous or non-proteinaceous. The nature and/or suitability of a particular modification can be routinely determined by the person of skill in the art.

For example, examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH4; amidination with methylacetimidate;

acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-

hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated herein is shown in Table 1.

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TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib

	D-valine	Dval	α-methyl- -aminobutyrate	Mgabu
	D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
	D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
	D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
5	D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
10	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
	D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
15	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
20	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
25	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
30	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen

	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
5	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ-aminobutyric acid	Gabu	N-(p-hydroxyphenyl)glycine	Nhtyr
	L-t-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
10	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L-α-methylalanine	Mala
	L-α-methylarginine	Marg	L-α-methylasparagine	Masn
	L-α-methylaspartate	Masp	L-α-methyl-t-butylglycine	Mtbug
	L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
15	L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
	L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
	L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
	L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
20	L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
	L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
	L-α-methylserine	Mser	L-α-methylthreonine	Mthr
	L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
	L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
25	N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
	1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

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Crosslinkers can be used, for example, to stablise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety.

Modulation of said activin functional levels may be achieved via the administration of said activin, a nucleic acid molecule encoding said activin or an agent which effects modulation of said activin activity or said activin gene expression (herein collectively referred to as "modulatory agents"). Preferably, the subject method is utilised to down-regulate the inflammatory response in a mammal.

Accordingly, in a particularly preferred embodiment the present invention is directed to a method of down-regulating the inflammatory response in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to induce a functionally ineffective level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in said mammal.

Preferably, said activin is activin A and/or activin B.

Preferably, said inflammatory response is modulated via modulation of the pro-inflammatory cytokine cascade. Still more preferably, said pro-inflammatory cytokine cascade is characterised by expression of TNF-α, IL-1 and/or IL-6.

Even more preferably, said agent is follistatin.

More preferably, said inflammatory response is an acute local inflammatory response or an acute systemic inflammatory response.

In accordance with these preferred embodiments of the present invention, said acute inflammatory response occurs in the context of, or is otherwise associated with, septic

shock, septicaemia, airway inflammation, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, or wound healing or traumatic injury such as occurs with surgery and burns.

Preferably, said airway inflammation occurs in the context of asthma, intersticial lung disease, cystic fibrosis, lung transplantation, bronchiolitis obliterans, emphysema, obstructive pulmonary disease, asbestosis, obstructive sleep apnoea, hypoxia or pulmonary hypertension.

Preferably, said acute systemic inflammatory response occurs in the context of systemic inflammatory response syndrome and even more particularly sepsis, septicaemia, toxic shock, septic shock, tissue trauma, meningitis or appendicitis.

In another preferred embodiment there is provided a method of up-regulating the inflammatory response in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to induce a functionally effective level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in said mammal.

Preferably said activin is activin A and/or activin B.

Preferably, said agent is the activin A or activin B expression product.

The term "mammal" as used herein includes humans, primates, livestock animals (eg. horses, cattle, sheep, pigs, donkeys), laboratory test animals (eg. mice, rats, guinea pigs), companion animals (eg. dogs, cats) and captive wild animal (eg. kangaroos, deer, foxes). Preferably, the mammal is a human or a laboratory test animal. Even more preferably, the mammal is a human.

Reference to "induce" should be understood as a reference to achieving the desired activin level, whether that be a functionally effective level or a functionally ineffective level. Said

induction is most likely to be achieved via the up-regulation or down-regulation of activin expression, as hereinbefore described, although any other suitable means of achieving induction are nevertheless herewith encompassed by the method of the present invention.

As detailed hereinbefore, a further aspect of the present invention relates to the use of the invention in relation to the treatment and/or prophylaxis of disease conditions or other unwanted conditions.

The present invention therefore contemplates a method of therapeutically and/or prophylactically treating a condition, or a predisposition to the development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal, said method comprising modulating the level of activin in said mammal where up-regulating activin to a functionally effective level up-regulates the pro-inflammatory mediator cascade and down-regulating activin to a functionally ineffective level inhibits or retards the pro-inflammatory mediator cascade.

More particularly, the present invention therefore contemplates a method of therapeutically and/or prophylactically treating a condition or a predisposition to the development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal, said method comprising modulating the level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in said mammal wherein upregulating said activin to a functionally effective level up-regulates the pro-inflammatory cytokine cascade and down-regulating said activin to a functionally ineffective level inhibits or retards the pro-inflammatory cytokine cascade.

Preferably, said activin is activin A and/or activin B.

Preferably, said pro-inflammatory cytokine cascade is characterised by the expression of TNF-α, IL-1 and/or IL-6.

Reference to an "aberrant, unwanted or otherwise inappropriate" inflammatory response

should be understood as a reference to an excessive response, an inadequate response or to a physiologically normal response which is inappropriate in that it is unwanted or otherwise inappropriate. Examples of aberrant or otherwise unwanted inflammatory responses include those which occur in the context of septic shock, septicaemia, airway inflammation, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, or wound healing or traumatic injury such as occurs with surgery and burns. In this regard, however, some forms of airway inflammation in fact reflect normal physiological responses which are unwanted, such as those which occur in the context of allergy or asthma. Examples of inadequate responses include the failure of any significant inflammatory response to occur as part of an immunisation regime.

Accordingly, the subject inflammatory response is preferably an unwanted acute inflammatory response of either the local or systemic type.

There is therefore preferably provided a method of therapeutically and/or prophylactically treating a condition, or a predisposition to the development of a condition, characterised by an unwanted acute inflammatory response in a mammal, said method comprising down-regulating the level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in said mammal wherein down regulating said activin to a functionally ineffective level inhibits or retards the pro-inflammatory cytokine cascade.

Preferably, said activin is activin A and/or activin B.

In accordance with this preferred embodiment of the present invention, said condition is septic shock, septicaemia, airway inflammation, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, or wound healing or traumatic injury such as occurs with surgery and burns.

Preferably, said airway inflammation occurs in the context of asthma, intersticial lung

disease, cystic fibrosis, lung transplantation, bronchiolitis obliterans, emphysema, obstructive pulmonary disease, asbestosis, obstructive sleep apnoea, hypoxia or pulmonary hypertension.

More preferably, said condition is systemic inflammatory response syndrome and even more particularly sepsis, septicaemia, toxic shock, septic shock, tissue trauma, meningitis or appendicitis.

In another preferred embodiment there is provided a method of therapeutically and/or prophylactically treating a condition, or a predisposition to the development of a condition, characterised by an inadequate inflammatory response in a mammal, said method comprising modulating the level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in said mammal wherein up-regulating said activin to a functionally effective level up-regulates the pro-inflammatory cytokine cascade.

Preferably, said activin is activin A and/or activin B.

These therapeutic and prophylactic aspects of the present invention are preferably achieved by administering an effective amount of a modulatory agent, as hereinbefore defined, for a time and under conditions sufficient to appropriately modulate the pro-inflammatory cytokine cascade.

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of the particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of the individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Preferably, to the extent that one is seeking to down-regulate an inflammatory response, said agent is follistatin.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

The present invention further contemplates a combination of therapies, such as the administration of the modulatory agent together with other proteinaceous or non-proteinaceous molecules which may facilitate the desired therapeutic or prophylactic outcome. For example, one may combine the method of the present invention with radiotherapy or chemotherapy.

Administration of molecules of the present invention hereinbefore described [herein collectively referred to as "modulatory agent"], in the form of a pharmaceutical composition, may be performed by any convenient means. The modulatory agent of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the modulatory agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 μg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

The modulatory agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), respiratory, transdermal, intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). The modulatory agent may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

Routes of administration include, but are not limited to, respiratorally, transdermally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraoccularly, intrathecally, intracereberally, intranasally, infusion, orally, rectally, via IV drip, patch and implant. Preferably, said means of administration is inhalation with respect to the treatment of airway inflammation and intravenously, intramuscularly or transdermally for other conditions.

In accordance with these methods, the agent defined in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. For example, the subject agent may be administered together with an agonistic agent in order to enhance its effects. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

In accordance with the present invention, although the preferred method is to therapeutically treat unwanted acute inflammatory responses, in certain circumstances one

may also seek to treat chronic inflammatory conditions. It is appreciated that achieving the down-regulation of a chronic inflammatory response is unlikely to reverse any tissue remodelling (scar formation) which has already occurred. However, such a method could prevent the occurrence of any further tissue damage. With respect to the prophylactic applications of the present invention, there are many circumstances where one may wish to institute a preventative treatment regime. For example, one may institute such a regime in patients who are predisposed to developing an autoimmune condition, patients who have suffered a tissue trauma such as severe burns, patients undergoing an organ transplant, cystic fibrosis patients, asthma/allergy sufferers or those prone to breathing disorders such as sleep apnoea.

Another aspect of the present invention relates to the use of an agent capable of modulating the functionally effective level of activin in the manufacture of a medicament for the therapeutic and/or prophylactic treatment of a condition, or a predisposition to the development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal wherein up-regulating activin to a functionally effective level up-regulates the pro-inflammatory mediator cascade and down-regulating activin to a functionally ineffective level inhibits or retards the pro-inflammatory mediator cascade.

More particularly, the present invention relates to the use of an agent capable of modulating the functionally effective level of activin, which activin is activin A or an activin molecule comprising a β_B subunit, in the manufacture of a medicament for the therapeutic and/or prophylactic treatment of a condition, or a predisposition to the development of a condition, characterised by an aberrant, unwanted or otherwise inappropriate inflammatory response in a mammal wherein up-regulating said activin to a functionally effective level up-regulates the pro-inflammatory cytokine cascade and down-regulating said activin to a functionally ineffective level inhibits or retards the pro-inflammatory cytokine cascade.

Preferably, said inflammatory response is an acute inflammatory response of either the

acute or systemic type.

In accordance with these preferred aspects of the present invention, said acute inflammatory response is preferably down-regulated and said condition is septic shock, septicaemia, airway inflammation, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, or wound healing or traumatic injury such as occurs with surgery and burns and said inflammatory response is down-regulated.

Preferably, said airway inflammation occurs in the context of asthma, intersticial lung disease, cystic fibrosis, lung transplantation, bronchiolitis obliterans, emphysema, obstructive pulmonary disease, asbestosis, obstructive sleep apnoea, hypoxia or pulmonary hypertension.

Preferably, said acute systemic inflammatory response occurs in the context of systemic inflammatory response syndrome and even more particularly sepsis, septicaemia, toxic shock, septic shock, tissue trauma, meningitis or appendicitis.

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents. Said agents are referred to as the active ingredients

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and

vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the

present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The agent may also be prepared for administration via the airway in either a particulate or soluble form. For example, the agent may be administered via an oral inhaler or a nebuliser.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule encoding activin A or a modulatory agent as hereinbefore defined. The vector may, for example, be a viral vector.

The present invention is defined by the following non-limiting examples.

EXAMPLE 1

Materials and Methods

Animals and general experimental details.

All experiments were conducted in accordance with the NHMRC Australian Code of Practice for the Care of Animals for Scientific Purposes (1997) and were approved by the Monash University Animal Ethics Committee.

One hundred and twenty six male C57Bl/6 mice (4-8 weeks), were randomly allocated into two groups; Group 1 consisted of nine sub-groups of eight animals (total n = 72) while Group 2 consisted of nine subgroups of six animals (n = 54). All animals were kept in standard animal housing with access to food and water throughout the experiment. Lipopolysaccharide (LPS) (*E. coli* serotype 0127:B8, Sigma, St. Louis, MO, USA) was purified using a phenol-water extraction method as previously described (Manthey *et al.* 1994, *J Immunol* 153:2653-63), and administered as an intraperitoneal bolus injection of 100 μg in 100 μl of isotonic, non-pyrogenic saline solution per mouse. Recombinant human follistatin-288 (rhfollistatin-288; Biotech, Australia) was administered as an intraperitoneal injection of 1 μg in 100 μl of isotonic, non-pyrogenic saline solution, 30 minutes prior to LPS. Group 1 received injections of LPS and rhfollistatin-288 while Group 2 received an injection of LPS alone. Mice were then anaesthetized with an inhalant form of isoflurane (Abbott Australasia LTD, Kurnell, Australia), and sacrificed for blood collection at 30 minutes, 1, 2, 3, 5, 8, 12 and 24 hours and one group was sacrificed without an injection to act as controls for basal levels. Blood was collected into a 1.5 ml centrifuge tube containing 50μl of ethylene diaminetetraacetic acid (EDTA, BDH Laboratory Supplies, Poole, UK) and centrifuged at 250*g* at room temperature with plasma removed and stored at –20°C until assayed for activin A, follistatin, TNFα, IL-6 and IL-1β.

Assays

Activin A was measured by ELISA as previously described using human recombinant activin A as a standard (Knight *et al.*, *J Endocrinol* 148:267-79). This ELISA measures both free and follistatin-bound activin and does not cross react significantly with other isoforms of activin (Knight *et al.*, *supra*). The mean sensitivity was 0.01 ng/ml, and the mean intra- and inter-assay coefficients of variations (CVs) were 3.9% and 5.1% respectively.

Follistatin concentrations in serum were measured with a radioimmunoassay as previously described (O'Connor *et al.*, *Hum Reprod.* 14:827-832). The standard and tracer employed was rhfollistatin-288. As with the Activin A ELISA, this RIA measures both free and bound forms of follistatins. The mean assay sensitivity was 2.7 ng/ml. ED_{50} was 13.3 ng/ml, and the intra- and inter-assay CVs were 6.4% and 10.2%, respectively.

Mouse cytokines TNFα, IL-6 and IL-1β were measured by ELISA (R&D Systems, Minneapolis, MN, USA). These assays use mouse recombinant proteins as standards and monoclonal antibodies for detection. The sensitivity of TNFα assay was 0.5 ng/ml, and the intra- and inter-assay CVs were <10%. The sensitivity of the IL-6 assay was 0.2 ng/ml and the intra- and inter-assay CVs were <10% and 12% respectively. The sensitivity of the IL-1β was ng/ml and the intra- and inter-assay CVs were <10% and <11%, respectively.

Data Analysis

All data was analysed using a one way ANOVA with a paired t-test used to compare differences between time points in the different treatment groups.

Results

The role of activin A in mice following an intraperitoneal LPS challenge

A robust release of activin A was observed in the mice following an injection of re-extracted LPS. Levels of activin A increased within 30 minutes following LPS administration and peaked at 1 hour returning to baseline levels between 3 to 8 hours, followed by a subsequent increase at 12 hours before returning again to baseline levels at 24 hours (Fig. 1A). Following LPS administration, follistatin was released into the circulation but was delayed compared to activin A, increasing at 3 hours and remaining elevated until 24 hours (Fig. 1B). The release of TNFα into the circulation was observed to follow the classic monophasic peak, increasing at 0.5 hours ($p<0.01$) post LPS administration, peaking at 1 hour and returning to basal levels at 5 to 8 hours (Fig. 1C). Serum IL-6 was elevated subsequent to elevations in TNFα, increasing between 1 and 2 hours, peaking at 2 hours ($p<0.01$) and remaining elevated until between 5 ($p<0.01$) and 8 hours (Fig. 1D). The levels of IL-1β in the circulation were significantly lower than TNFα or IL-6 (30-50 fold) with IL-1β increasing 1 hour post injection and peaking at 5 hours ($p<0.01$) before returning to basal levels at 8 hours (Fig. 1E).

The peak release of activin A was unaffected by the administration of rhfollistatin-288. Following LPS administration activin A release into the circulation was still rapid and robust peaking at 1 hour and returning to basal levels within 5 hours (Fig. 2A). Interestingly, the concentration of circulatory mouse follistatin-288 was significantly suppressed over the entire peak period, 5-8 hours ($p<0.03$) following LPS administration in mice injected with rhfollistatin-288 (Fig. 2B). Additionally, TNFα release was significantly suppressed by administration of rhfollistatin-288 prior to injection of LPS ($p<0.01$) although the profile of release was not significantly altered (Fig. 2C). Conversely, IL-6 release was altered in both absolute amounts and temporally. Interestingly, IL-6 peak concentrations were significantly increased ($p<0.01$) in mice administered rhfollistatin-288 prior to LPS by approximately 2 fold (Fig. 2D). Furthermore, increases in IL-6 occurred earlier in the presence of rhfollistatin-288, peaking

at 1 hour as compared to 2 hours in mice receiving LPS alone. Release of IL-1β was not as evident in the presence of rhfollistatin-288 when compared to mice that received LPS alone (Fig. 2E). Additionally, the profile also shifted such that elevations in serum concentrations occurred earlier in the presence of rhfollistatin-288, peaking at 2 hours compared to 5 hours in mice receiving LPS alone ($p<0.01$). However, it should be noted that there was not a significant difference in the concentrations of IL-1β at any time point.

EXAMPLE 2

ACTIVIN AND FOLLISTATIN IN A MOUSE MODEL OF EXPERIMENTAL ALLERGIC ASTHMA

Pilot data from our ovalbumin (OVA) sensitisation and challenge model of allergic asthma highlights major changes in activin expression during the evolution of the pulmonary inflammatory response.

Compartmentalisation of activin and follistatin is observed in a mouse model of allergic asthma, and activin expression in various cellular sites in lung tissue from asthmatic and cystic fibrosis patients (Figure 3). The kinetics of activin secretion have been mapped finding that the peak concentration in BALF (Fig 4A) coincides with peak inflammation and eosinophilia (Fig 4B), and the production of IL-4 (Fig 4C).

Immunohistochemical analysis of activin expression in the lung shows that activin is expressed in airway epithelium from control (saline) mice (Fig 5A). However, after 4 OVA challenges (day 8) the airway undergo profound changes, with epithelial cell hypertrophy and marked loss of activin expression (Fig 5B). These alterations persist until day 17 (10 days after final challenge), although activin expression becomes variable between adjacent airway and even within the same airway (Fig 5C). Collectively, these findings indicate that pre-stored activin is released into the surrounding tissue during the inflammatory response. A general trend toward normal airway morphology and activin expression at the later time points suggests that this remodelling process is reversible. Finally, preliminary

immunohistochemical analysis reveals loss of follistatin expression in bronchial epithelium after OVA challenge very similar to the pattern seen for activin.

EXAMPLE 3

CHARACTERISATION OF PULMONARY EXPRESSION OF ACTIVIN AND FOLLISTATIN

Activin and follistatin mRNA expression and activin receptor compartmentalisation in the mouse

Using a sensitisation and challenge protocol with OVA as allergen we have found a correlation between magnitude of the inflammatory response and differential regulation of activin and follistatin expression in bronchial epithelium versus BALF has been found. The finding that activin protein is dramatically decreased in bronchial epithelial cells mandates that activin and follistatin expression should be assessed at multiple time-points during and following the immunisation protocol. Mice are sensitised with OVA (50 µg in aluminium hydroxide) on days 0 and 12, and challenged via intratracheal intubation with OVA (25 µg) on days 24, 26, 28 and 30 (Hardy *et al.*, 2003, *Am J Respir Crit Care Med* 167:1393-1399). Control mice receive saline instead of OVA. Mice are killed (n = 6 per group) after each of 4 allergen challenges, and on days 2, 4, 7, 10 and 20 after the final OVA challenge. Immunohistochemistry is performed on formalin-fixed lung. Activin and follistatin are detected with specific antibodies (E4, raised against the human activin ßA subunit; 2E6, raised against human recombinant follistatin) which cross-react with mouse; isotype matched antibodies serve as controls. Primary antibodies are detected with appropriate anti-mouse–horseradish peroxidase antibodies. Measurement of activin A in BALF and serum is according to an established enzyme linked ELISA protocol (Knight *et al.*, 1996, *supra*) using human recombinant activin A standard. Follistatin concentration in BALF and serum are measured using a discontinuous radioimmunoassay (O'Connor *et al.*, 1999, *supra*). An established real-time RT-PCR protocol is used to quantitate activin and follistatin mRNA in lung tissue. Immunohistochemistry (Santa Cruz Biotechnology) is also used to evaluate expression of type I and II activin receptors to determine which cells

might be responsive to activin. In a smaller number of time-points non-radioactive *in situ* hybridisation is performed to determine the localisation of activin receptor mRNAs to measure any change in the compartmentalisation of mRNAs concomitant with the shift in protein localisation. Activin and follistatin staining intensity in epithelium and bronchial submucosa is scored using double blind analysis on a scale of 0 = absent, 1 = weak, 2 = moderate, and 3 = high intensity. Ten bronchioles of internal diameter 150–200 μm from each mouse are analysed to arrive at scores for individual mice.

Activin and follistatin expression in human airway disease

A detailed immunochemical analysis of activin and follistatin expression in normal, asthmatic and cystic fibrosis lung tissue and BAL (see methods above) is performed. Tissue specimens are obtained from stored and prospective lung tissue samples resected at the time of transplantation (severe cystic fibrosis n=20), with the co-operation of the Heart Lung Transplantation Service, The Alfred Hospital, Melbourne. Asthmatic tissue are available from stored resected lung tissue and prospective endobronchial biopsy tissue from asthmatic patients undergoing bronchoscopy for intercurrent diagnostic reasons (n=10). Age-matched control airway from non-smokers with no known history of airway disease are collected from fresh *post mortem* specimens provided by The Department of Anatomical Pathology (n=20). Tissue is collected from proximal airway (right lower lobe bronchus) at the time of lung resection. Specimens are fixed in each of: (1) chilled acetone with protease inhibitors at –20°C for later embedding in glycol methacrylate (GMA), and (2) ethanol and formalin for subsequent paraffin embedding.

EXAMPLE 4

DEFINING THE RELATIONSHIP BETWEEN ACTIVIN AND FOLLISTATIN EXPRESSION AND PULMONARY INFLAMMATION

Key aspects of the allergic inflammatory response are measured in order to characterise the relationship between activins and follistatin and magnitude of the inflammatory response. Mice (n = 6 per group) are sensitised and challenged with OVA (as outlined in Aim 1), and

killed after each of 4 allergen challenges, and on days 2, 4, 7, 10 and 20 after the final OVA challenge. Serum is collected from whole blood, and tested for presence of OVA-specific IgE and IgG_1 by sandwich ELISA. Lung tissue is fixed in formalin prior to paraffin embedding; sections are stained with haematoxylin and eosin and periodic acid-Schiff for microscopic assessment of inflammation and for determination of mucus-producing cell frequency. BAL and mediastinal lymph node single-cell suspensions are counted. BAL cell cytospots are Giemsa stained and differential counts performed on $\geq$ 200 cells per mouse; cells are identified by morphologic criteria. Frequency of IL-4, IL-5, IL-13 and IFN-γ producing cells in OVA-stimulated mediastinal lymph nodes are determined by ELISPOT (BD Biosciences and R&D Systems). ELISPOT plates are read on an AID ELISPOT Reader. BALF is collected after cell counts have been performed, and stored at –70°C for subsequent analysis of the above cytokines by sandwich ELISA. Additionally, formalin-fixed lung tissue from control, asthmatic and cystic fibrosis patients is immunohistochemically stained to detect mast cells (AA1, Dako), eosinophils (eosinophil major basic protein, eosinophil peroxidase, BD Biosciences), T lymphocytes (CD3, Dako) and macrophages (CD68, PGM1, Dako). Cells are counted using a 3-layer amplification system with streptavidin biotin-peroxidase and AEC (Sigma-Aldrich) as the substrate. Counts are performed using an image analyser (Image-Pro Plus, MediaCybernetics) to a depth of 150 µm below the basement membrane and expressed as cells per mm^2. The expression of key Th2 cytokines are measured in BALF from patients and controls (BD Biosciences).

EXAMPLE 5

CORRELATING ACTIVIN AND FOLLISTATIN EXPRESSION WITH AIRWAY REMODELLING

Remodelling events in stored and prospective samples from normal (n = 20), asthmatic (n = 10) and cystic fibrosis (n = 20) human lung are analysed. Morphometric image analysis and immunohistochemistry are used to measure key indices of the remodelling response including: (i) thickening of the sub-epithelial basement membrane, (ii) fibroblast proliferation, (iii) myofibroblast hyperplasia, (iv) airway smooth muscle

hypertrophy/hyperplasia, and (v) angiogenesis. Sub-epithelial basement membrane thickness and angiogenesis are measured using well established protocols (Li *et al.*, 1997, *Am J Respir Crit Care Med* 156:229-233; Wilson *et al.*, 1997, *Clin Exp Allergy* 27:363-371; Orsida *et al.*, 1999, *Thorax* 54:289-295). Airway smooth muscle hypertrophy and hyperplasia are assessed on haematoxylin & eosin stained sections (Image-Pro Plus) by measuring smooth muscle cell diameter in μm (diameter across the nucleus) and percentage smooth muscle in the bronchial submucosa (Benayoun *et al.*, 2003, *Am J Respir Crit Care Med* 167:1360-1368). Additionally, airway smooth muscle hypertrophy are assessed immunohistochemically by scoring intensity of α-smooth muscle actin and myosin light chain kinase expression (Sigma-Aldrich) on a scale of 0–3 (see Aim 1) (Benayoun *et al.*, 2003, *supra*). Fibroblast proliferation is assessed immunohistochemically on formalin-fixed sections using antibody specific for proliferating cell nuclear antigen (PCNA, Dako). Fibroblasts are identified using morphological criteria and staining for prolyl-4-hydroxylase (Dako). The number of PCNA-positive fibroblasts below the basement membrane are counted, normalised to the basement membrane length and expressed per mm^2 of quantifiable biopsy area (Image-Pro Plus). All parameters are measured on at least 2 serial sections for each patient.

EXAMPLE 6

INVESTIGATING WHETHER FOLLISTATIN TREATMENT PREVENTS PULMONARY INFLAMMATION AND ENHANCES RESOLUTION IN A MURINE MODEL

Follistatin modulation of activin expression and release – murine model of acute asthma

Activin function is regulated by a number of binding proteins, the best studied being its interaction with the high-affinity binding protein follistatin. Binding to human recombinant follistatin effectively blocks interaction with the activin receptor, thereby neutralising the biological actions of activin A (Phillips, 2000, *Bioessays* 22:689-696). Using mouse asthma models, the ability of follistatin to modulate activin expression and release in lung, BAL and serum is assessed, comparing different follistatin doses and routes of

administration. Intraperitoneal injection of 1 μg follistatin per adult mouse 0.5 hour prior to LPS injection blocks the rise in follistatin seen 4 hours later, and suppresses release of proinflammatory cytokines (TNF-α and IL-1β), while activin release is unimpaired. Thus follistatin treatment blocks activin-induced effects, but not its release. Initially, mice (n = 6 per group) receive i.p. 1 μg follistatin per mouse, 0.5 hour prior to each of the four OVA challenges. This route of follistatin delivery is compared with intranasal and intratracheal administration testing varying doses and timings of administration. Control mice receive saline. Activin A and follistatin expression is monitored by ELISA in BALF, and by RT-PCR and immunohistochemistry in the lung (see Aim 1) following each of the four OVA challenges, and on days 2, 4, 7, 10 and 20 after the final OVA challenge. The latter time point reveals whether activin A expression returns to pre-challenge levels in untreated OVA mice, and gives an indication as to the duration of the follistatin-induced blockade of activin.

Secondly, a determination if made of whether neutralisation of activin A by follistatin lessens the severity and duration of the allergic pulmonary inflammation. The ability of follistatin to attenuate pulmonary inflammation by measuring key 'allergic' parameters including specific IgE and IgG_1, eosinophilia, mucus hypersecretion, and cytokine production is investigated. Mice are killed following each of the four OVA challenges, and on days 2, 4, 7, 10 and 20 after the final OVA challenge. Blood, BAL, lungs and mediastinal lymph nodes are collected for enumeration of inflammatory cells, OVA-specific IgE and IgG_1, eosinophilia, mucus production and ELISPOT analysis of IL-4, IL-5, IL-13, and IFN-γ. (methods as per Aim 1). Since TGF-ß is also involved in immunoregulation and tissue remodelling, TGF-ß concentration in BALF is measured (R&D Systems) and TGF-ß expression in tissue sections is measured by immunohistochemistry (Santa Cruz Biotechnology) to determine whether its production is modulated by activin/follistatin (Lee *et al.*, 2001, *J Exp Med* 194:809-821). These data provide information regarding the ability of activin neutralisation to ameliorate allergic pulmonary inflammation.

Follistatin modulation of activin expression and release – murine model of chronic asthma

Repeat antigen dosing at non-tolerogenic time intervals for up to six weeks is performed in a murine model of chronic asthma (2 challenges/week on Monday and Thursday) to induce sustained airway inflammation and chronic remodelling (Coyle *et al.*, 1996, *J Immunol* 156:2680-2685). Mice are treated with follistatin according to the dose and route optimised above. The effects of follistatin treatment on remodelling in this mouse model are assessed by measuring: (i) sub-epithelial basement membrane thickening, (ii) angiogenesis, (iii) smooth muscle hypertrophy, and (iv) mucus cell induction (Lee *et al.*, 2001, *supra*; Kumar *et al.*, 2002, *Clin Exp Allergy* 32:1104-1111). Sub-epithelial basement membrane thickness, angiogenesis and smooth muscle hypertrophy are assessed. Metaplasia and/or hyperplasia of mucus-secreting goblet cells is assessed. These data provide information regarding the ability of follistatin to inhibit the airway remodelling response.

Statistical analysis

The distribution of each data set is tested for normality before analysis. Normally distributed data is analysed using one-way ANOVA with Bonferroni's correction for multiple comparisons. Individual comparisons between groups is made using a two-tailed Student's t-Test. Relationships between activin/follistatin expression and either inflammation or remodelling indices is analysed using Pearson's correlation.

Data that are not normally distributed are analysed using the non-parametric Kruskal-Wallis Test followed by Dunn's Multiple Comparisons post-hoc test. Individual comparisons between groups are made using a two-tailed Mann-Whitney U-Test for non-parametric data. Relationships between activin/follistatin expression and either inflammation or remodelling indices are explored using Spearman's rank correlation. A P value of ≤ 0.05 will be considered significant.

EXAMPLE 7

PROFOUND CHANGES IN ACTIVIN β_B DURING LOCALISED AND SYSTEMIC INFLAMMATION

Materials and Methods

Experimental design

For the systemic LPS model, male C57/BL mice were injected intraperitoneally (ip) with 100 µg phenol-purified LPS (Sigma: *E.Coli* (0127:B8). Control mice, injected with PBS, were sacrificed at time 0 and remaining animals (6/time point) at 0.5, 1, 3, 5, 8, 12 and 24 hours and following LPS injection. In an independent experiment, the effects of activins were neutralized by the pretreatment of mice with the activin binding protein, follistatin, which is able to bind and ablate the effects of activin forms [Nakamura *et al.*, 1990, *Science* 247:836-838]. In this experiment, mice were pretreated ip with human recombinant follistatin 288 (1 µg) 30 minutes prior to an injection of LPS. Mice were sacrificed 30 minutes after the follistatin injection (time 0) and at the same times, relative to LPS, as indicated above. At the time of sacrifice, tissues to be examined for expression levels were placed in ice cold Trizol (Invitrogen Life Technologies) and stored at –80 C for later RNA extraction. Tissues were also placed in formalin prior to transfer to 70% ethanol for later fixation and immunohistochemical studies.

For the acute hepatic inflammation model, male C57/BL6 mice were injected ip with 750 µl/kg BW CCl_4 (Sigma). Control mice, injected with PBS, were sacrificed at time 0 and remaining animals were sacrificed at 1, 2, 4,8, 12, 24, 36, 48 and 72 hours following CCl_4 injection. Tissues were collected as described earlier for RNA extraction and immunohistochemical studies.

RNA extractions were performed on 3-5 tissue samples from each time point described above. RNA was extracted using Trizol according to the manufacturer's recommendations. For each sample, approximately 10 µg of RNA was treated with DNAse I (Ambion Inc.) in

accordance with the manufacturer's protocol. RNA concentrations for each sample were determined and 1 μg was reverse transcribed to give cDNA using Superscript III reverse transcriptase kit (Invitrogen Life technologies) and using the protocol supplied by the manufacturer. Real time analysis for expression levels were made for the following genes: GAPDH, activin β_A subunit, and activin β_B subunit. Inhibin α-subunit mRNA expression was also examined using standard thermocycler methods but expression levels were consistently too low to permit quantitative analysis (data not shown).

The specific primers utilized for the real-time quantification of the genes were (5'- to 3'):

GAPDH	F tactggcatcttcaccacca (Product 394 bp)	(SEQ ID NO:1)
activin β_A	Fggctaacagaaccaggacca (Product 325bp)	(SEQ ID NO:2)
activin β_B	F gacacgcatagccagactca (Product 399bp)	(SEQ ID NO:3)
inhibin α-subunit	F cttatgtattccggccatcc (Product 326bp)	(SEQ ID NO:4)
GAPDH	R gtgagcttcccattcagctc (Product 394 bp)	(SEQ ID NO:5)
activin β_A	R cttcttcccatctccatcca (Product 325bp)	(SEQ ID NO:6)
activin β_B	R acttgccctctccaagaaca (Product 399bp)	(SEQ ID NO:7)
inhibin α-subunit	R cctagtgtgggctaccagga (Product 326bp)	(SEQ ID NO:8)

The primers were designed specifically for use with the Roche light cycler real-time PCR system. PCR products were isolated and sequenced and BLAST analysis used to confirm they represented the desired gene products. Real time analyses were conducted using Roche SYBR green mastermix (Light cycler Fast start DNA Master SYBR green , Roche Diagnostics GmbH) with conditions optimised for maximal sensitivity. Annealing temperatures for all primers were 60 C. Standards and QCs used throughout the analyses were prepared from pooled cDNA derived from experimental samples in which expression levels of the genes of interest were high. Serial dilutions of the standard cDNA was to cover a 300-fold expression range. Experimental cDNA samples were diluted into the standard curve range and all cDNA was aliquoted and stored at –20. Each sample was analysed for all three gene products of interest, at least twice in independent analytical runs. Between assay QC reproducibility for all gene products gave CVs of < 22%.

Immunohistochemistry

Paraffin sections were dewaxed and antigens retrieved by immersing slides in 0.01M citrate buffer, pH 6.0, heating in a microwave (high for 2.5 minutes or 5 minutes for βA or βB respectively, then low for 5 minutes for both), cooling at 4 C for ~20 minutes, and washing in water for 5 minutes. Endogenous peroxidase was blocked in 3% H_2O_2 for 10 minutes, and slides blocked for 1 hour (10% normal rabbit serum + CAS block, Zymed Laboratories Inc., CA, # 00-8120) for activin βA or 20% normal goat serum/0.1% Tween 20 in Tris-buffered saline (TBS) for 1 hour for activin βB. The blocking solution was tapped off and the sections were incubated with antibodies specific for the activin βA-subunit (E4, 10 μg/ml in 1% bovine serum albumin (BSA)/TBS, Oxford Brookes University) or activin βB subunit (2 μg/ml diluted in blocking solution, Jones *et al.* 2000) overnight at 4 C. After washing, the activin βA slides were incubated in rabbit anti-mouse IgG_{2b}-HRP (Zymed, # 61-0320) diluted 1:500 for 2 hours and washed twice in Tris-buffered NaCl (TBS) 0.05% Tween-20 pH 7.5, then MilliQ H_2O. Reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zymed # 00-2014), and sections counterstained in hematoxylin for 15 seconds. All wash steps were in TBS/0.05% Tween-20. For the activin βB slides, the sections were washed and then incubated with Dako Envision HRP (rabbit, #K4003) for 1 hour at room temperature. The sections were washed again in TBS/Tween and the reaction product was developed with DAB, followed by counterstaining as for activin βA. Negative control sections were incubated with purified mouse myeloma IgG2B protein (Zymed #02-6300) instead of the activin βA-specific antibody or non-immunized rabbit IgG (Dako #X0903) instead of the activin βB-specific antibody.

Data analysis

For each sample, activin βA and βB mRNA expression levels were expressed relative to the GAPDH expression level for that sample. Thereafter, all time 0 data were normalized to 1 and data at subsequent time points was expressed relative to that time point. All data

are depicted as mean ± SEM values. Values were typically derived from the 3 tissue samples assessed per time point, but more samples were assessed in controls and at some early time points.

Results

In a mouse model of acute systemic inflammation following challenge with LPS, liver mRNA levels for the activin βA and βB subunits were examined. Activin βA subunit mRNA showed a minor increase (<2-fold control levels) in expression level 1 hour after LPS, but between 1 and 3 hours expression levels fell markedly and from 3 to 8 hours a clear suppression (to <25% of control levels) was evident (Figure 6, upper panel). By 12 hours, expression was approaching control levels and by 24 hours had returned to pretreatment levels. Treatment with the activin binding protein and antagonist, follistatin, resulted in an immediate suppression in activin βA subunit mRNA levels.

In contrast, liver βB subunit mRNA levels displayed a completely different profile to the activin βA subunit, rising immediately after LPS to reach a maximal expression level at 5 hours, at which time, expression averaged over 35-fold control levels (Figure 6, lower panel). Between 5 and 12 hours this expression fell progressively but at 12 hours, expression was still elevated (on average, 7-fold control levels). At 24 hours after LPS treatment, activin βB mRNA levels were still ~5- fold above control levels. As for activin βA subunit expression, activin βB subunit expression patterns were altered by the follistatin pretreatment, with clear suppression of the LPS-associated effects on βB subunit expression.

In the acute hepatic inflammation model following challenge with CCl_4, activin βA subunit expression fell slightly following CCl_4 treatment (Figure 7, upper panel), such that at 1 and 2 hours, average expression levels were only 40-50% of control levels. In contrast, by 4 hours after injection, average βA mRNA was moderately (80%) elevated and then declined to around pre-treatment levels by 36 hours. In contrast to the activin βA subunit, activin βB

showed the greatest changes in expression at 24 and 36 hours after CCl_4 injection, with a 13.5-fold increase above control levels (Figure 7, lower panel).

In both inflammatory models, expression of the inhibin α-subunit was examined but expression levels were consistently too low to permit quantitative analysis. Therefore it is unlikely that the profound changes in activin βB subunit mRNA resulted in the formation of elevated inhibin dimers (an α-βB dimer or inhibin B), but dimerized to form activin B (a dimer of βB-βB). Given the only marginal changes in activin βA mRNA, it is relatively unlikely that the increased βB mRNA expression resulted in significant formation of the heterodimer, activin AB (βA-βB).

Using antibodies specific for the activin βA and βB subunits, the liver immunolocalization was investigated in both the acute systemic model of LPS challenge and the acute hepatic inflammatory model using CCl_4. Localization of the activin βA subunit in normal liver was in hepatocytes and more specifically those predominantly around the central veins (Figure 8). Following LPS challenge, the localization appeared to diminish around 5 hours after LPS and returned to a pre-treatment distribution by 12 hours. For the activin βB subunit, however, localization was most evident in hepatocytes surrounding the portal tract areas of the liver and less so around the central veins (Figure 9). However, the localization appeared to diminish at 5 hours following LPS and returned to pre-treatment patterns by 12 hours. There also appeared to be a loss of hepatocyte localization in peripheral areas of the liver (Figure 9). In the CCl_4 model of acute hepatic inflammation, subunits localized to the hepatocytes surrounding the central vein and portal tracts for the βA and βB subunits respectively (Figure 10). However, 36 hours after CCl_4 treatment, there appeared to be localization for the activin βA subunit in hepatocytes that were destined to become apoptotic/necrotic, whereas there was no or little localization for the activin βB subunit in these areas (Figure 10).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also

includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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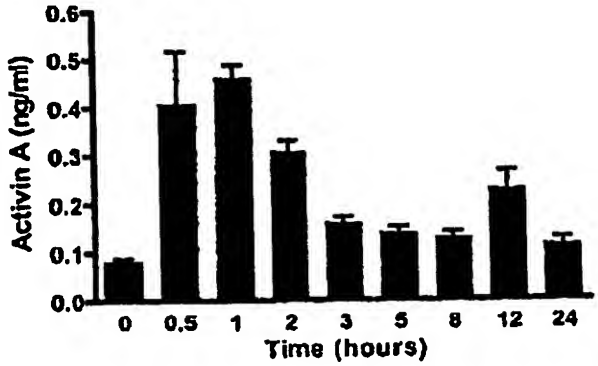
DATED this 24th day of August, 2004

Monash University
by DAVIES COLLISON CAVE
Patent Attorneys for the Applicant(s)

Figure 1A



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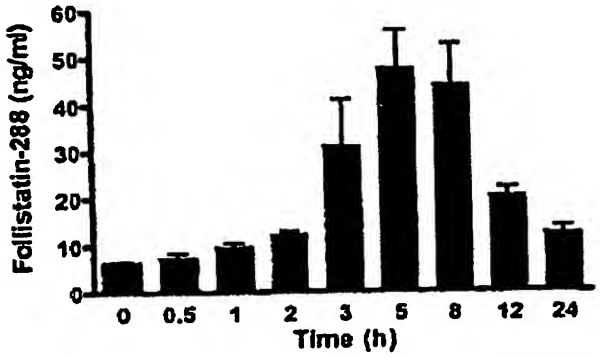

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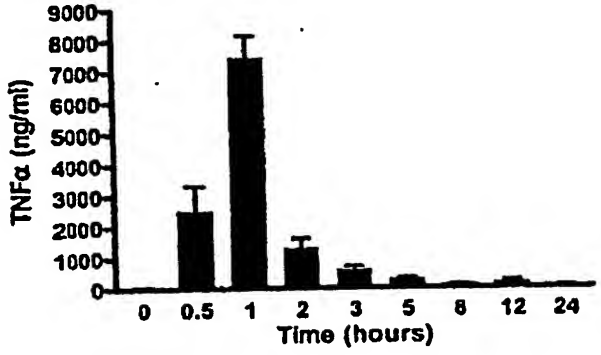

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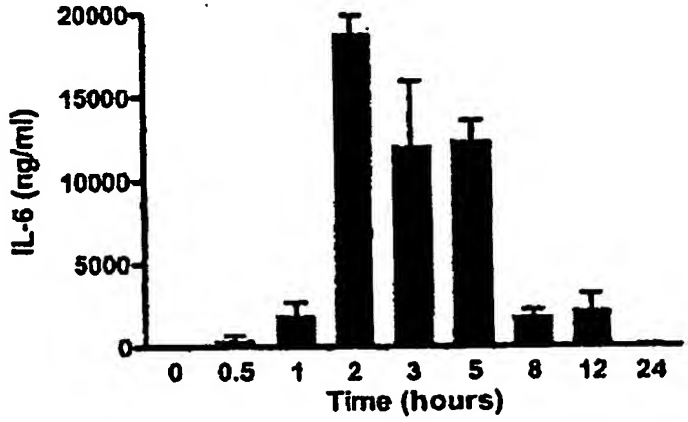

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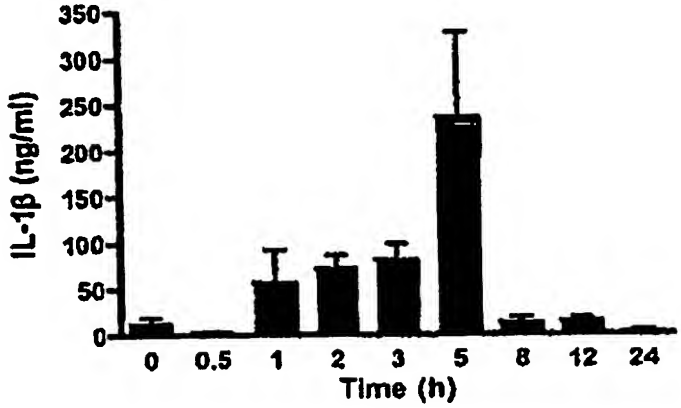

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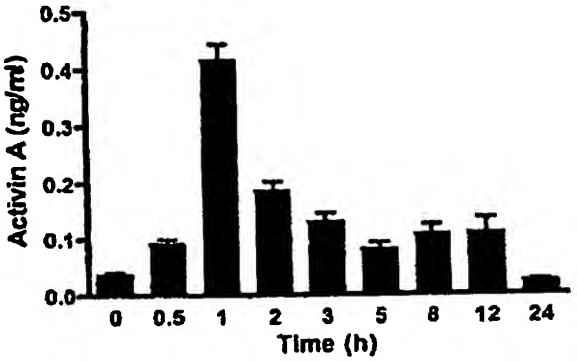

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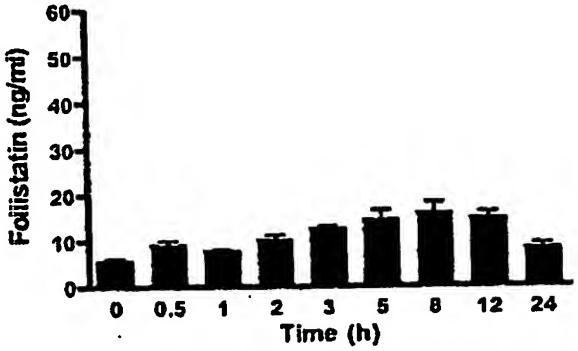

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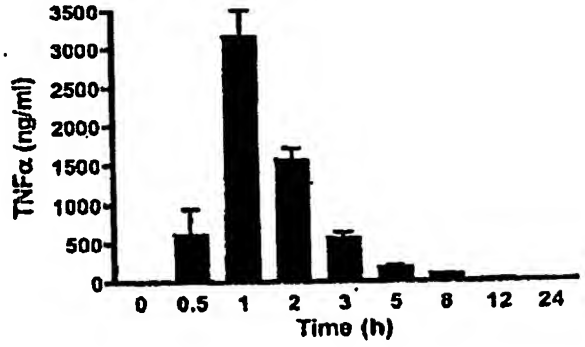

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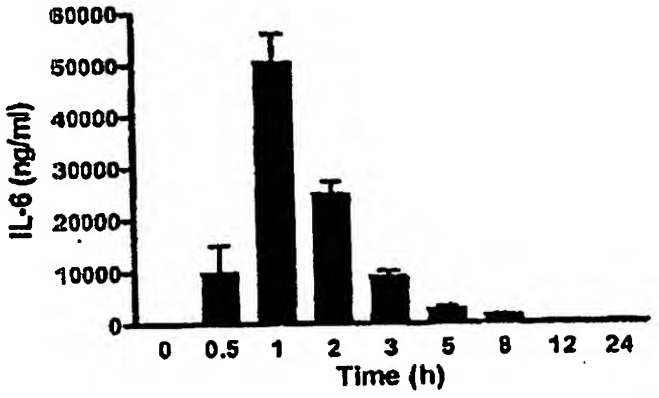

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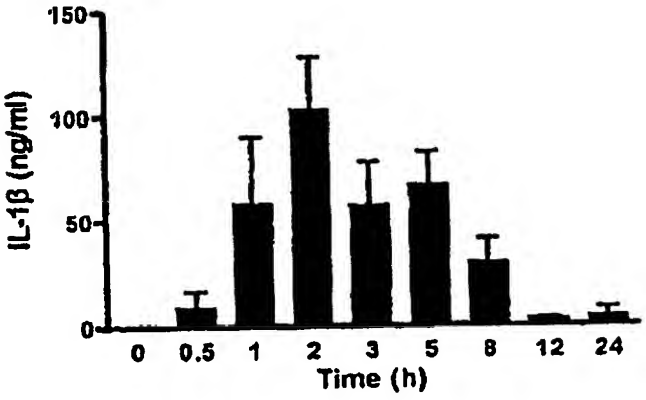
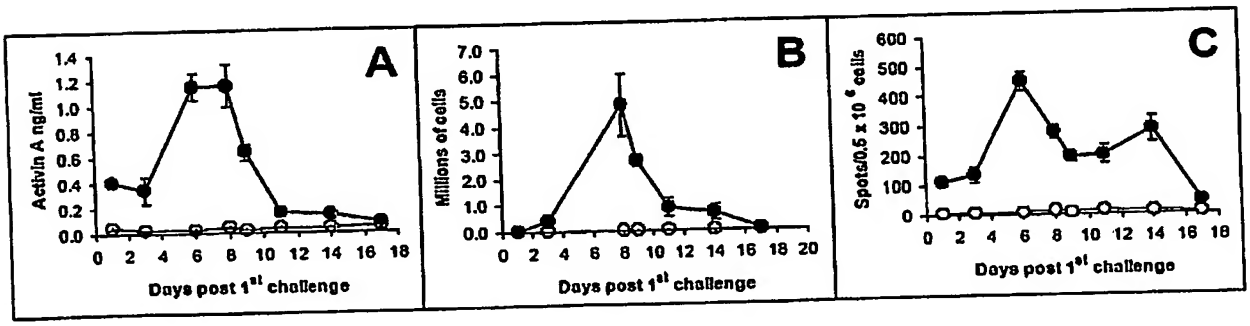


Figure 3

Figure 4



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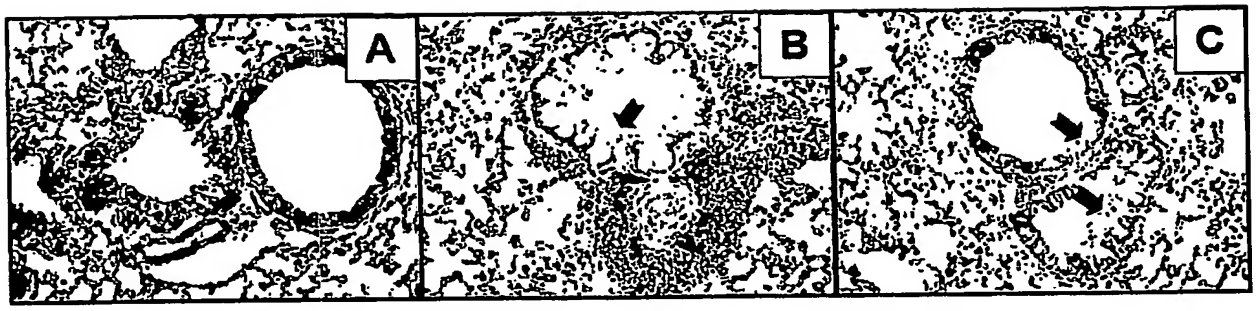

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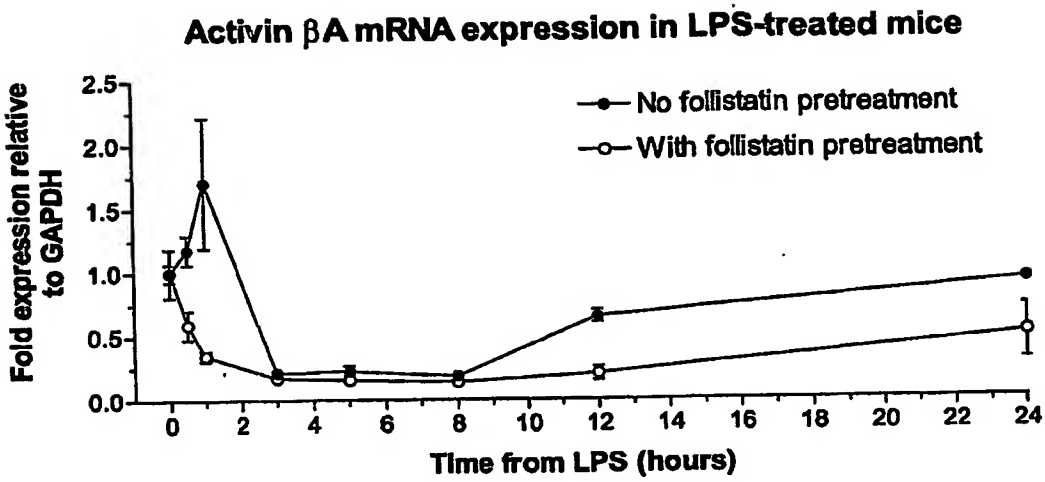

Activin βA mRNA expression in LPS-treated mice
Fold expression relative to GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time from LPS (hours)
No follistatin pretreatment
With follistatin pretreatment

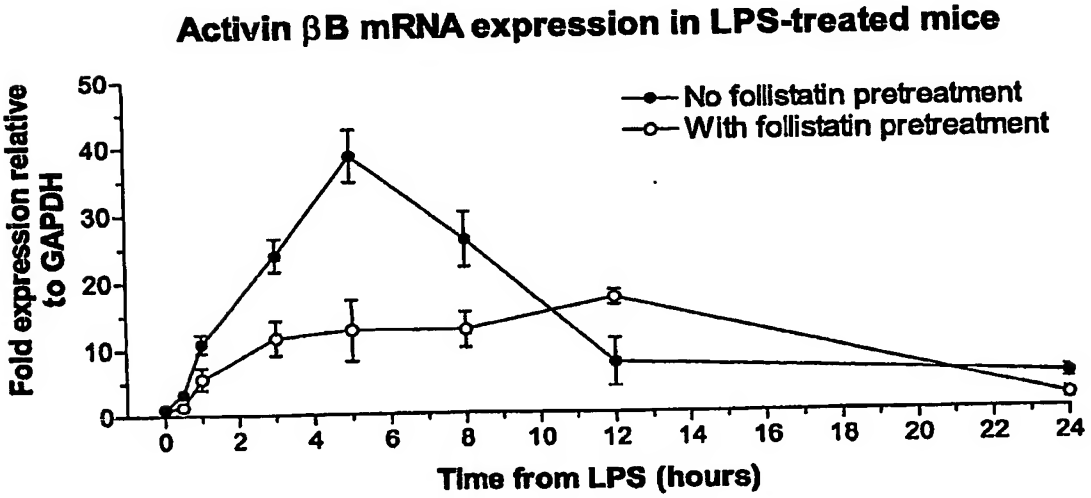
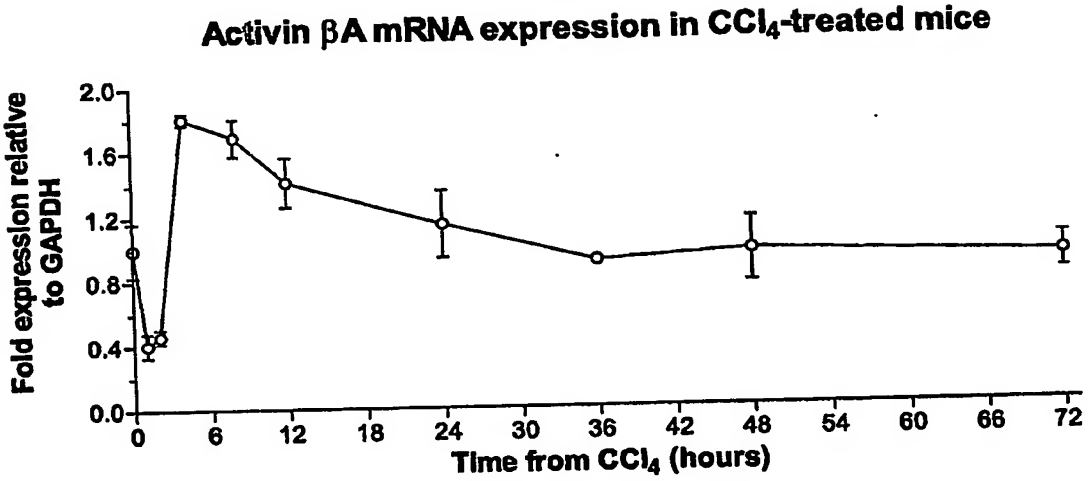

Activin βB mRNA expression in LPS-treated mice
Fold expression relative to GAPDH
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time from LPS (hours)
No follistatin pretreatment
With follistatin pretreatment

Figure 7


Activin βA mRNA expression in CCl4-treated mice
Fold expression relative to GAPDH
2.0
1.6
1.2
0.8
0.4
0.0
0
6
12
18
24
30
36
42
48
54
60
66
72
Time from CCl4 (hours)

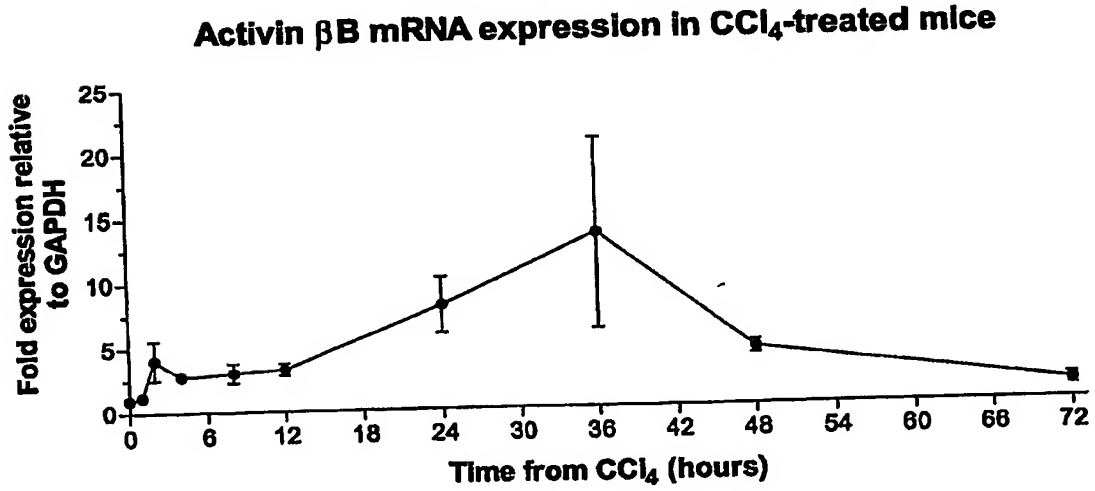
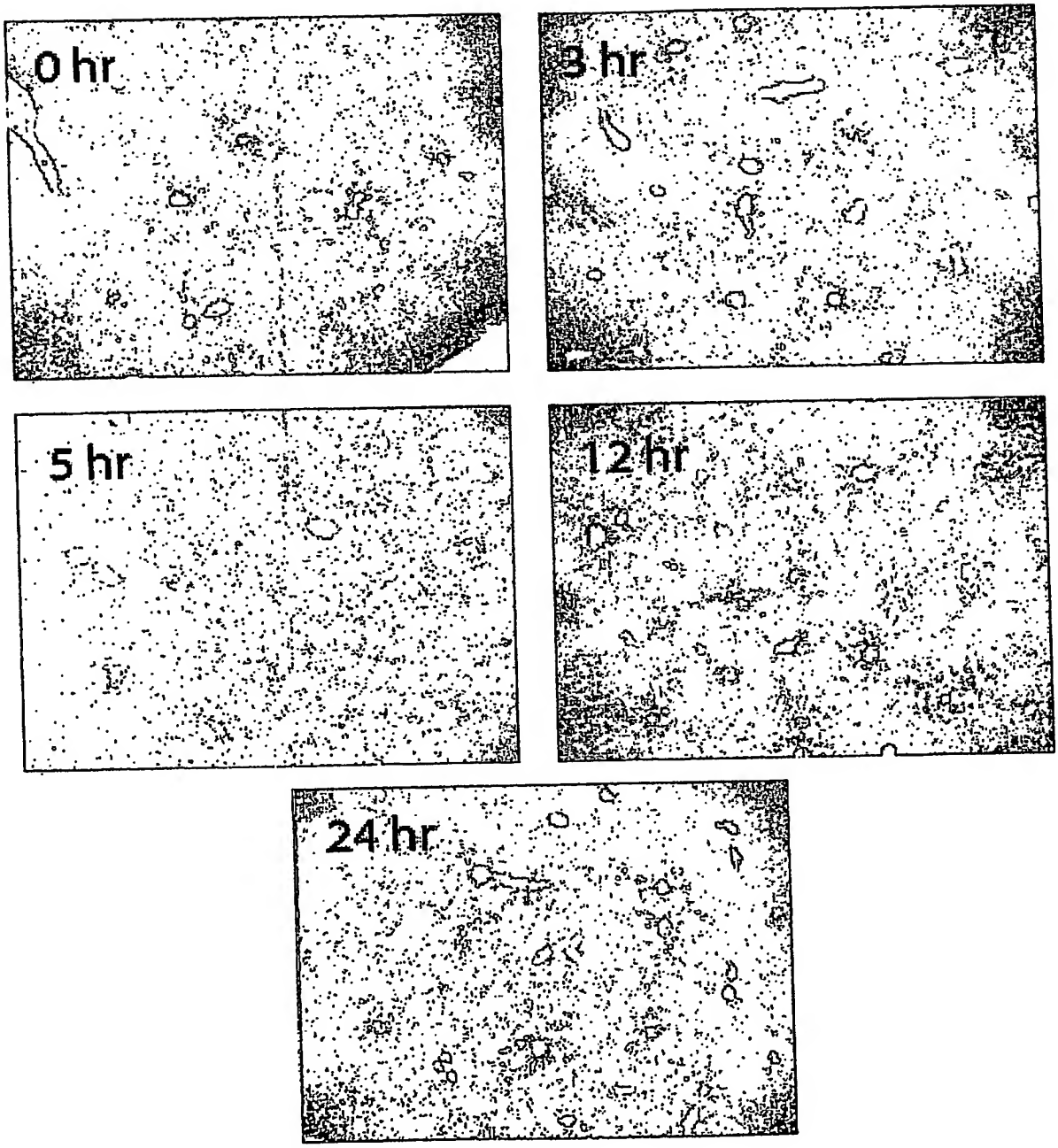

Activin βB mRNA expression in CCl4-treated mice
Fold expression relative to GAPDH
25
20
15
10
5
0
0
6
12
18
24
30
36
42
48
54
60
66
72
Time from CCl4 (hours)

Figure 8



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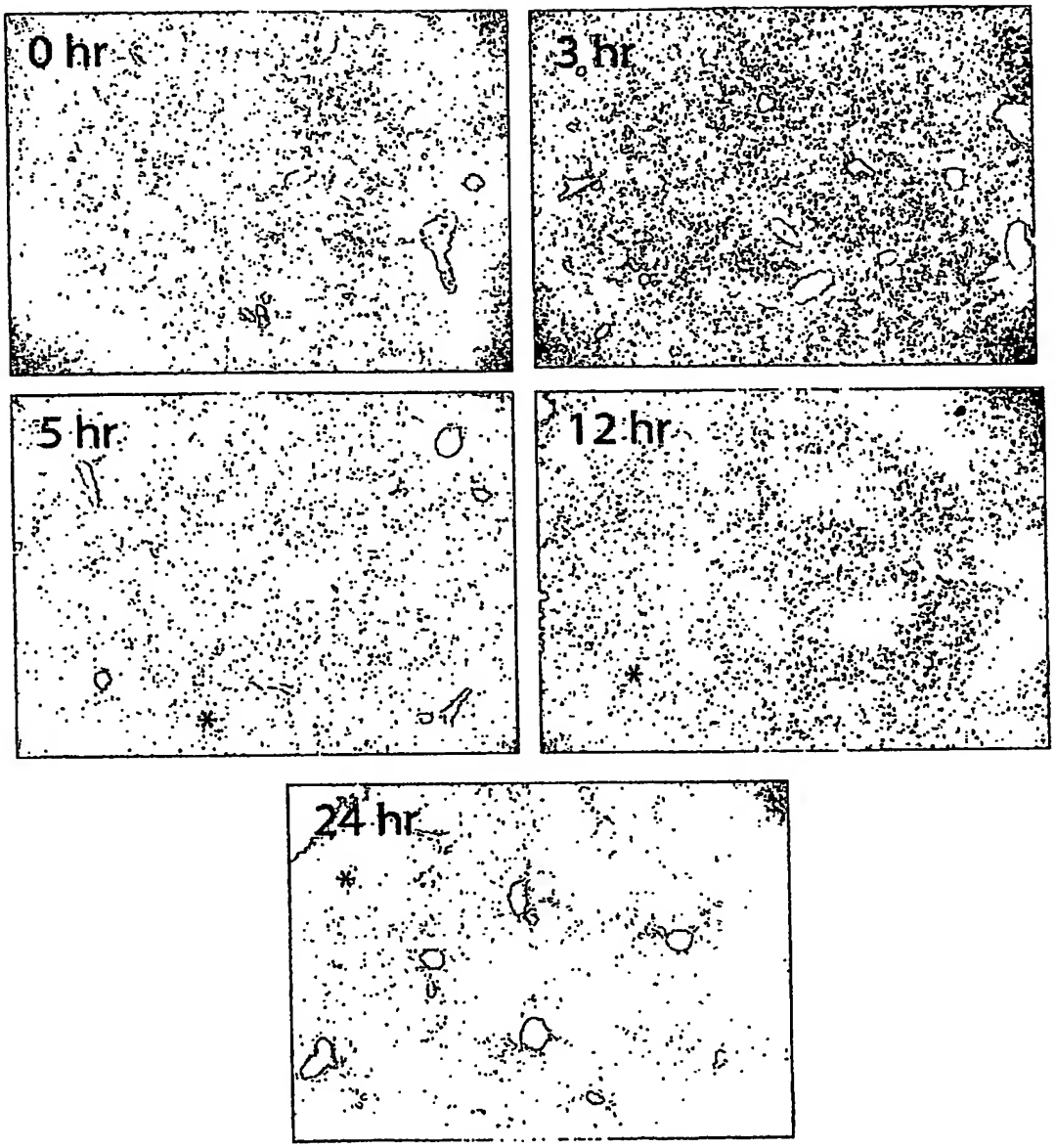

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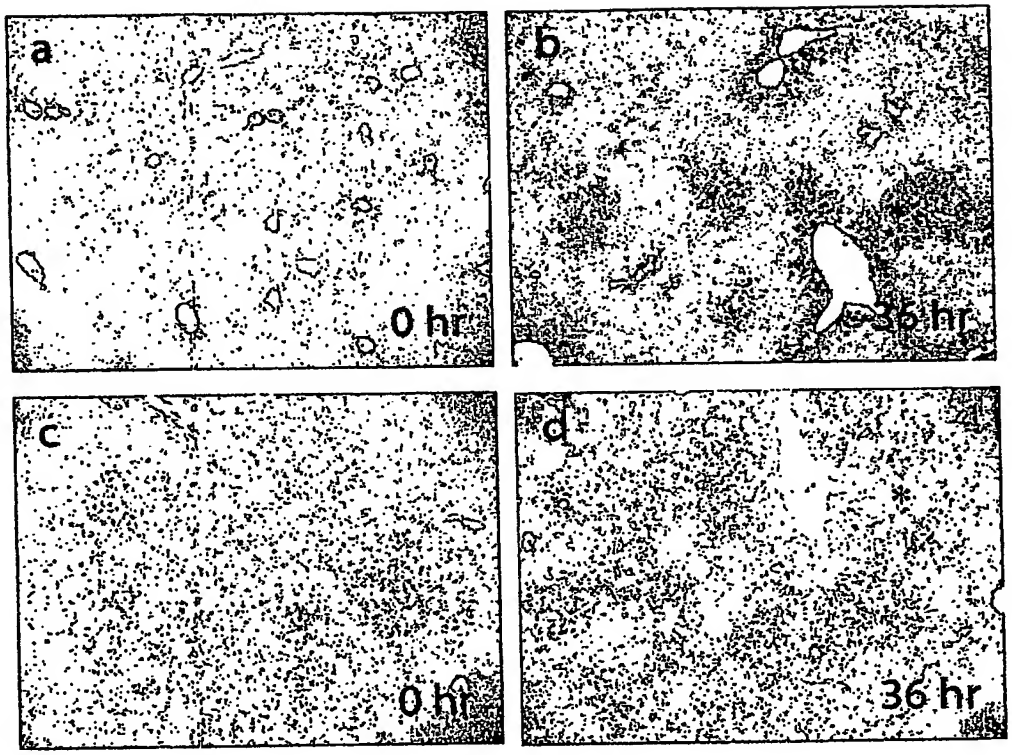

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